TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 0061/00091

U.S. APPLICATION NO. (If known, see 37 CFR 1.5): 09/622257

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/FR99/00316	12 February 1999	13 February 1998

TITLE OF INVENTION

NUCLEIC ACID COMPRISING THE SEQUENCE OF A STRESS-INDUCIBLE PROMOTER AND A SEQUENCE OF A GENE ENCODING A STILBENE SYNTHASE

APPLICANT(S) FOR DO/EO/US

COUTOS-THEVENOT, Pierre, HAIN, Rüdiger, SCHREIER, Peter-Helmut, BOULAY, Michel

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. § 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3).

9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. ☒ A translation of the Annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification.

15. ☐ A change of power of attorney and/or address letter

16. ☒ Other items or information:

Copy of the International Preliminary Examination Report, with amended sheets and an **English translation of the amended sheets**; copy of the sequence listing on paper in French with an English translation thereof; copy of the sequence listing in computer-readable format (i.e., on diskette); copy of PCT/IB/308; copy of the International Search Report; copy of PCT/IB/306

Error! Bookmark not defined. U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 09/622257	INTERNATIONAL APPLICATION NO. PCT/FR99/00316	ATTORNEY'S DOCKET NUMBER 0061/00091

534 Rec'd PCT/PTO 25 AUG 2000

☒ The following fees are submitted:

	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)):		
Search Report has been prepared by the EPO or JPO.....$840.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) $670.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)).....$760.00		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....$970.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....$96.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$840.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	**$130.00**	

Claims	Number Filed	Number Extra	Rate		
Total Claims	29 - 20 =	9	X $18.00	**$162.00**	
Independent Claims	1 - 3 =	0	X $78.00	**$0.00**	
Multiple dependent claim(s)(if applicable)			+ $260.00	**$0.00**	
TOTAL OF ABOVE CALCULATIONS =				**$1,132.00**	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28)				**$0.00**	
SUBTOTAL =				**$1,132.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				**$0.00**	
TOTAL NATIONAL FEE =				**$1,132.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				**$0.00**	
TOTAL FEES ENCLOSED =				**$1,132.00**	
				Amount to be: refunded	**$**
				charged	**$**

a. ☒ A check in the amount of $1,132.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. **22-0185** in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Director is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **22-0185**. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b) must be filed and granted to restore the application to pending status

SEND ALL CORRESPONDENCE TO:

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Morris Liss
SIGNATURE

Morris Liss
NAME

24,510
REGISTRATION NUMBER

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	:	
	:	
Pierre Coutos-Thevenot et al.	:	
	:	
Serial No.: To be assigned	:	Art Unit: To be assigned
	:	
Filed: Herewith	:	Examiner: To be assigned
	:	
For: NUCLEIC ACID COMPRISING THE SEQUENCE OF A STRESS-INDUCIBLE PROMOTER AND A SEQUENCE OF A GENE ENCODING A STILBENE SYNTHASE	:	Atty Docket: 0061/00091

PRELIMINARY AMENDMENT

Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to initial examination, please amend the above-captioned case as follows.

IN THE CLAIMS

From the **amended sheets**, please amend claims 3, 6, 9, 12, 13, 20, 22, 23, 24, 25, and 27, and add new claim 29 as follows:

At claim 3, line 1, replace "one of Claims 1 and 2" with --Claim 1--.

At claim 6, line 1, replace "one of Claims 1 to 5" with --Claim 1--.

At claim 9, line 3, replace "one of Claims 1 to 8" with --Claim 1--.

At claim 12, lines 1-2, replace "one of Claims 9 to 10" with --Claim 9--.

At claim 13, lines 1-2, replace "one of Claims 9 to 12" with --Claim 9--.

At claim 20, line 2, replace "one of Claims 9 to 19" with --Claim 9--.

22. (Amended) Process for obtaining a cell according to [one of Claims 20 and 21, characterized in that] Claim 20, wherein a plant cell is transformed using a microbiological method including a system [or vector according to one of Claims 9 to 19] for expressing a stilbene synthase gene in plants comprising at least one nucleic acid.

At claim 23, line 4, replace "one of Claims 9 to 19" with --Claim 9--.

At claim 24, line 2, replace "one of Claims 9 to 19" with --Claim 9--.

At claim 25, lines 1-2, replace "one of Claims 20 to 21" with --Claim 20--.

At claim 26, line 2, replace "one of Claims 22 to 23" with --Claim 22--.

At claim 27, line 1, replace "one of Claims 24 to 26" with --Claim 24--.

--29. Process for obtaining a cell according to Claim 21, wherein a plant cell is transformed using a microbiological method including a vector for expressing a stilbene synthase gene in plants comprising at least one nucleic acid.--

REMARKS

The claims have been amended to eliminate multiple dependency and to improve their format. None of these amendments is believed to involve any new matter. Accordingly, it is respectfully requested that the foregoing amendments be entered, that the application as so amended receive an examination on the merits, and that the claims as now presented receive an early allowance.

Respectfully submitted,

Morris Liss

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8/14/00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No. :

U.S. National Serial No. :

Filed :

PCT International Application No. : PCT/FR99/00316

VERIFICATION OF A TRANSLATION

I, Susan POTTS BA ACIS

Director to RWS Group plc, of Europa House, Marsham Way, Gerrards Cross, Buckinghamshire, England declare:

That the translator responsible for the attached translation is knowledgeable in the French language in which the below identified international application was filed, and that, to the best of RWS Group plc knowledge and belief, the English translation of the international application No. PCT/FR99/00316 is a true and complete translation of the above identified international application as filed.

I hereby declare that all the statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the patent application issued thereon.

Date: 17 July 2000

Signature of Director :

For and on behalf of RWS Group plc

Post Office Address : Europa House, Marsham Way,
Gerrards Cross, Buckinghamshire,
England.

NUCLEIC ACID COMPRISING THE SEQUENCE OF A STRESS-INDUCIBLE PROMOTER AND A SEQUENCE OF A GENE ENCODING A STILBENE SYNTHASE

The present invention relates to plants which exhibit improved resistance to certain pathogenic agents which are sensitive to stilbenes, and relates, more specifically, to a set of constructs which combine a plant promoter which can be induced by a biotic stress, which stress is engendered, in particular, by the said pathogens, with (a) gene(s) encoding a stilbene synthase.

A large part of the world harvest of cultivated plants is regularly destroyed by parasites and pathogens. Among the possible options for decreasing or preventing the attack by these parasites on cultivated plants, chemical control (plant protection treatments) is the method which is most used. Nevertheless, the application of chemical products is not without consequences for the environment and sometimes presents technological problems as, for example, the appearance of new resistant pathogenic strains or, in the field of oenology, the difficulties which can arise during fermentation (the use of inhibitors of sterol biosynthesis can block yeast growth at the end of fermentation) or the presence of chemical products, such as procymidone, an anti-Botrytis product, which are sometimes found in wine.

The control method which consists in improving the resistance of cultivated plants to the diseases which are caused by these pathogens has been envisaged as a way of overcoming the drawbacks associated with chemical control. It is possible, for example, in a first approach, to achieve this improvement by the sexual route, i.e. using classical genetics, by hybridizing the plants whose resistance is to be improved with tolerant varieties. Nevertheless, this approach is not always feasible (tolerant natural variety not known) or is not permitted by legislation

such as, for example, in viticulture as a result of French legislation on Appellations d'Origine Controlée (A.O.C.) (registered designations of origin) which limits the grapevine varieties which are to be used for a given appellation (designation).

In a second approach, it is possible, using the modern techniques of cell and molecular biology, to integrate, into the genome of the plant, one or more homologous or heterologous genes which make it possible to overexpress or express a molecule of interest, which is of protein nature, in order to increase the production of a metabolite, or a metabolic pathway, or to open a new biosynthetic pathway or to synthesize a novel molecule for example for increasing the opening of a new biosynthetic pathway, for example increasing the resistance of the plant by reinforcing its defence mechanisms with regard to the pathogens in question.

There are several different defence mechanisms of this type in plants. Some can be regarded as being passive and are linked to the physicochemical characteristics of the cells, the epidermal tissues and/or the organs of the plant. Others belong to the dynamics of gene/gene interactions (plant resistance genes and pathogen avirulence genes, mechanisms of host/pathogen interactions). While these interactions can lead to the development of a hypersensitivity reaction (rapid death of the cells of the plant around the point of infection in order to block colonization of the plant by the microorganism), they can also lead to the synthesis and accumulation of a whole series of compounds. Of these, some can be parietal constituents which are involved in the formation of a "physical" barrier around the point of infection (callose, lignin, hydroxyproline-rich protein: HRGP, etc.), and other compounds can be molecules having antimicrobial functions which are more or less well defined (phytoalexins, pathogen-associated proteins: PR proteins (pathogenesis-related proteins), etc.). The molecules of the phytoalexin type which are synthesized and

accumulated by plants during, for example, host/pathogen interactions include, in particular the stilbenes, which are toxic, in particular for microorganisms. The term stilbene designates a group of chemical substances which possess the trans-diphenyl-1,2-ethylene skeleton as the common basal structure, with resveratrol and pinosylvine being among the simplest. This basal skeleton is synthesized in plants by a stilbene synthase or related enzymes from substrates such as malonyl-CoA, cinnamoyl-CoA or coumaroyl-Coa, which are substances which are present in all plants (flavonoid precursors). Genes for stilbene synthase or related enzymes have been isolated, sequenced and cloned, in particular from groundnut, orchid and grapevine. Using these genes, it has been possible to transform plants such as potato, lucerne or tobacco, with these plants then exhibiting greater resistance than untransformed plants to pathogen attack (EP-309862; EP-648839; MELCHIOR, F. et al., Arch. Biochem. Biophys. 1991, 288, 2, 552-557; WIESE, W. et al., Plant Mol. Biol. 1994, 26,2,667-677; HAIN, R. et al., Nature 1993, 361, 153-156).

The expression or overexpression of these molecules having antimicrobial functions can provide plants with a "natural" resistance in response to stresses, in particular stresses of the microbial type. However, constitutive overexpression of this type of protein necessarily has disadvantages for the plant (energy cost, slowing down of growth, etc.) (FISCHER, R. et al., The Plant Journal 1997, 11, 3, 489-498).

On the other hand, in some plants, such as grapevine or herbaceous plants, stilbenes are only found in some healthy tissues and at very low concentrations. Conversely following an infection or a lesion, these stilbenes increase strongly at the infected or damaged site, since the stilbene synthase genes are inducible under conditions of biotic or abiotic stress (for example wounds, ultraviolet rays, etc.).

Nevertheless, this regulation is rarely present in plants of agricultural interest or, when it is

present, it can be insufficiently effective. For example, studies on phytoalexin synthesis in the grapevine have demonstrated that the only healthy tissue in which stilbenes, including resveratrol, are present is healthy wood tissue. Stilbene is found to be present in the tissues of the grape berry when, on the one hand, the berry has been subjected to a stress such as attack by a pathogen (*Botrytis cinerea* for grey mould or *Plasmopora viticola* for grape downy mildew) and, on the other hand, only during the period up to the incipient ripening of the young fruit. By contrast, the concentration of the stilbene decreases strongly from incipient ripening to maturation. However, damage due, for example, to *Botrytis* is rarely encountered during the period up to incipient ripening but rather during the period close to maturation of the berry. For this reason, expression of the stilbene synthase gene has to be controlled with strong promoters which escape the natural regulation of the gene and which should be inducible, in particular by the stress itself. The present invention specifically relates to a promoter of this nature.

The present invention relates to nucleic acids which comprise the sequence of the promoter for a lucerne PR protein linked to at least one sequence of a gene encoding a stilbene synthase.

The invention relates, in particular, to nucleic acids according to the invention, characterized in that the promoter for a lucerne PR protein is a promoter which can be induced in plants, in a tissue-specific manner or not, by a biotic or abiotic stress.

The invention also relates to nucleic acids according to the invention, characterized in that the sequence of the promoter for a lucerne PR protein is selected from the group comprising:

a) the IND S1 sequence,

b) any sequence corresponding to a fragment of the IND S1 sequence and having a promoter sequence effect in plants.

The sequences of the promoter for a lucerne PR protein are preferred which exhibit at least 80% homology with the IND S1 sequence. Those sequences are particularly preferred which exhibit at least 90% or 95% homology with the said sequence.

The sequences of promoters for lucerne PR proteins according to the invention were obtained from regulatory sequences of genes for PR proteins by taking advantage of the incompatibility response (hypersensitivity reaction, HR) obtained in the host/parasite relationship between lucerne (*Medicago sativa*) and *Pseudomonas syringae* pv *pisi* for the purpose of isolating the regulatory sequences of genes which are responsible for this reaction.

When *Pseudomonas* attacks lucerne, the occurrence of a plant reaction is observed in the region adjacent to the necrosis caused by the bacterial infection.

Plant material was therefore removed following the bacterial attack in order to construct a cDNA library from the messenger RNAs which were produced in the regions adjacent to the necrosis. Amplification by polymerase chain reaction (PCR), using synthetic polynucleotides corresponding to motifs which are conserved in leguminous PR protein genes, enabled a radioactive probe to be obtained which was then used to select transcripts in the cDNA library. One of these (cDNA-PR7) was adopted since, after sequencing, it exhibited good homology with equivalent genes encoding PR proteins and known from other plants (cf. Figures 1 and 1a, depicting the general scheme of the method for isolating the promoter).

Analysis showed that it corresponded to a gene encoding a class 10 PR protein according to the VAN LOON (1994) classification. This gene was therefore designated Ms PR10-1 (*Medicago sativa* PR class 10 protein, clone 1). The isolated and cloned cDNA PR7 made it possible to obtain two probes due to the presence of an internal *Bam HI* site (B in Figure 1). These probes, EB and BE (E corresponding to the *Eco RI* site in Figure

1) named respectively 5' and 3', were used to screen a lucerne genomic library. Among the clones obtained which were recognised by the 5' and 3' probes, one of them, C15, was selected and sequenced (6.1 Kb). It itself also possesses, logically, a *Bam HI* site which made it possible to obtain two novel EB and BE fragments of 2.4 kb and 3.7 kb, named respectively E-B(g) and B-E(g), g indicating the genomic nature of the fragments obtained (see Figure 1a). Fragment E-B(g), which is located in 5' of the C15 clone, and which comprises the promoter and a portion of its Ms PR10-1 gene coding sequence, was inserted into the *Eco RI* and *Bam HI* sites of the bluescript plasmid. The plasmid was linearized by means of a *Pst I* site which is located upstream of *Bam HI* in fragment E-B(g). A deletion from 3' to 5' was carried out on this fragment until the IND S1 promoter sequence was obtained (Figure 3). A blunt end ligation made it possible to reposition another *Bam HI* site, internal to the C15 clone, at the end of the promoter sequence of the Ms PR10-1 gene. Under these conditions, 13 nucleotides of the coding sequence of the Ms PR10-1 gene, located upstream of this *Bam HI* site which is internal to the C15 clone, were thus integrated into the IND S1 promoter sequence: the whole can be isolated with an *Eco RI/Bam HI* digestion (see Example 1).

In the description, PMs PR 10-1 is also intended to mean any nucleic acid fragment of the IND S1 sequence with a promoter effect in plants and the IND S1 sequence with 13 nucleotides of the coding sequence of the Ms PR10-1 gene as described below.

The invention also relates to nucleic acids according to the invention, characterized in that the sequence of a gene encoding a stilbene synthase, whether homologous or heterologous, is selected from the genes isolated from groundnut, orchid, grapevine and pine genomes (EP-309 862, EP-464 461).

Of the said nucleic acids, preference is given to the nucleic acids which encode a grapevine stilbene synthase, in particular those described in the article

by HAIN, R. et al., Natrue 1993, 361, 153-156 and in that by WIESE, W. et al., Plant Mol. Biol., 1994, 26, 2, 667-677; the nucleic acid corresponding to the sequence *vst1* of the said articles is that which is most preferred.

The nucleic acids enabling the stilbene synthase gene(s) to be expressed will naturally be able to include, in particular, apart from the said gene(s), polyadenylation sequences at the 3' end of the coding strand and enhancer sequences from the said gene or from a different gene.

Naturally, the nucleic acid sequences will have to be adapted in order to ensure that the gene is actually read in the correct reading frame with the promoter and it will obviously be possible to foresee using, if necessary, several promoters of the same type as well as several enhancer sequences.

It is also possible to use the nucleic acids according to the present invention to express several stilbene synthase genes, either arranged in tandem or carried by different expression systems.

The nucleic acids according to the invention can be used to create expression systems in plants, which systems can be inducible and/or constitutive depending on the tissues or organs of the plant which are transformed (cf. Examples 2, 3 and 4).

The present invention therefore also relates to systems for expressing at least one stilbene synthase gene in plants, characterized in that they comprise at least one nucleic acid according to the invention. Of the systems according to the invention, preference is given to transformation vectors, particularly transformation vectors of the plasmid type. Advantageously, the said transformation vectors are characterized in that they can be transferred into *Agrobacterium* strains.

The stilbene synthase genes which are able to be expressed by the nucleic acids according to the present invention are placed under the control of the PMs PR10-1

promoter for the purpose of activating, in plants, mechanisms of resistance to pathogens which are sensitive to stilbenes, in particular to resveratrol, to pinosyl grapevine or to their glycosylated derivatives such as picein or to oligomers such as the viniferins. Parasites of this nature which are sensitive to stilbenes and which may be mentioned are *Botrytis cinerea, Plasmopora viticola, Eutypa lata*, etc.

Preference is given to those expression systems according to the invention which are characterized in that they can be induced in plants by a biotic or abiotic stress.

From the said biotic stresses according to the invention preference is given in particular to biotic stresses which are engendered by the attack of a parasite which is sensitive to stilbenes, such as a virus, a bacterium, a yeast, a fungus, in particular *Botrytis cinerea* or *Plasmopora viticola*.

From the said abiotic stresses according to the invention, preference is given in particular to abiotic stresses engendered by a mechanical wound such as that caused in particular by an insect or by a physical phenomenon such as wind or frost.

The present invention also relates to plant cells which are transformed with a system or a vector according to the invention. Advantageously, the said plant cells are grapevine cells.

The present invention also relates to processes for transforming plant cells using a microbiological method, including the systems or vectors according to the present invention.

The invention furthermore relates to processes for obtaining plants which express one (or several) stilbene synthase gene(s), characterized in that cells of the said plants are transformed using a system or a vector according to the invention, the cells expressing the said gene(s) are selected and a plant is regenerated from these cells.

The most frequently used transformation methods

which may be mentioned are, in particular, the methods which employ *Agrobacterium*, whether this be *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*, biolistic or any other techniques (electroporation, etc.).

These methods are known (REAM, W., 1989; NEGRETIU, I. and GHARTI-CHHETRI, G.B., 1991; CASSE-DELBART, F., 1996; STANFORD, J.C., 1990) and will not be described again in detail.

The technology, which in particular makes use of plasmid systems, enables a first transformation of a competent bacterial strain, in general *E. coli*, to be effected, which transformation enables the structure of the plasmids to be cloned and monitored. The strain is then used to transfer the recombinant plasmids into agrobacterial strains, which will then be used to transform the plant cells.

The plants comprising an expression system or cells according to the invention are part of the invention.

The plants which are obtained by implementing the processes according to the invention are also part of the invention.

Finally, the invention relates to plants according to the invention, characterized in that the plants are plants of agricultural interest, in particular grapevine plants.

Other characteristics and advantages of the constructs and the processes according to the present invention will be evident from the examples which follow.

Figure legends

Figures 1 and 1a: General scheme depicting the different steps in the method for isolating the inducible promoter PMs PR10-1, corresponding to the IND S1 sequence.

Figure 2: Representation of the various clones which were isolated and which correspond to the Southern blot,

which blot was hybridized with the 5' and 3' parts of cDNA-PR7, which parts were delimited by an internal *Bam HI* (B) site which was detected in this cDNA.

Restriction sites: E = Eco *RI*, B = *Bam HI*.

The values indicated in the figure are expressed in kb (kilo bases).

Figure 3: DNA sequence corresponding to the IND S1 sequence, which is the isolated genomic sequence of the inducible lucerne promoter PMs PR10-1.

Figure 4: DNA sequence corresponding to the sequence of a gene for grapevine stilbene synthase which is modified by adding an adaptor (modified *vst1*).

The modified part is depicted in italics.

The coding and noncoding (intron) parts are depicted in upper case and lower case letters, respectively.

Figure 5: DNA sequence comprising the sequence of the inducible promoter PMs PR10-1 (corresponding to the IND S1 sequence in lower case) linked to the sequence of a gene for grapevine stilbene synthase which is modified by adding an adaptor (modified *vst1*, corresponding to Figure 4). Between the two (framed in the sequence) are (end of the promoter in lower case and start of the gene containing the translation start codon in upper case) the 13 nucleotides which come from an internal sequence of the coding frame of the Ms PR10-1 gene; since the ATG has been positioned in reading frame with the modified *vst1* gene, these nucleotides are thus integrated into the coding frame of *vst1*.

The sequence of the inducible promoter PMs PR10-1 includes the 7 of the 13 nucleotides of the sequence of the gene Ms PR10-1 (in lower case in the frame).

The coding and noncoding (intron) parts of the part corresponding to the sequence of the gene for grapevine stilbene synthase are depicted in upper case and lower case letters, respectively.

Figure 6: Demonstration of the induction of the gene encoding a stilbene synthase with UV light.

The RNAs are extracted from approximately 1 g of leaves 17 hours after inducing with UV light. From 10 to 20 µg are loaded onto a formaldehyde/formamide denaturing gel. After migration (3 $V.cm^{-1}$), the RNAs are transferred to a nylon membrane and fixed by exposure to UV light (254 nm, 33 $mJ.cm^{-2}$). The Northern blot is obtained by hybridizing, at 65°C and overnight, with the biotinylated probe *vst1*.

The explant which is used as the starting material consists of leaves which have been isolated from 41B (control) vitroplants or 41B vitroplants which have been genetically transformed with a construct (13 kb insert comprising two complete stilbene synthase genes, *vst1* and *vst2*, a fragment of grapevine genomic DNA and another truncated grapevine stilbene synthase gene (*vst3*)) which integrates supernumary copies of genes encoding grapevine stilbene synthase under the control of their own promoters (clones 2 and 3 corresponding to clones 55-2 and 55-3, respectively). 41B: stock-vine hybrid *V. vinifera, Chasselas H V. berlandierii*.

Figure 7: Kinetics of stilbene synthase mRNA accumulation following induction with UV light.

The RNAs are extracted from approximately 1 g of leaves. From 10 to 20 µg are loaded onto a formaldehyde/formamide denaturing gel. After migration (3 $V.cm^{-1}$), the RNAs are transferred to a nylon membrane and fixed by exposure to UV light (254 nm, 33 $mJ.cm^{-2}$). The Northern blot is obtained by hybridizing, at 65°C and overnight, with the biotinylated probe *vst1*.

The explants are leaves which have been isolated from 41B vitroplants. The control is a clone which has not been transformed genetically, contrary to clones 2 and 3 (corresponding to clones 55-2 and 55-3, respectively), which have integrated, into their genome, the 13 kb insert (see above) containing genes encoding grapevine stilbene synthases (*vst1* + *vst2*). 41B: stock-vine hybrid *V. vinifera, Chasselas H V. berlandierii*.

Figure 8: Quantity of resveratrol present in vitroplant

leaves which have been treated with UV light for 8 min and analysed at different periods after induction.

The quantities are expressed in µg per g of fresh material.

NI: not induced

The control consists of leaves taken from untransformed 41B. PCT 55-2 and 55-3 are two transformants which have integrated the 13 kb insert comprising two complete stilbene synthase genes (*vst1* + *vst2*).

Figure 9: Inhibition of the growth of *Botrytis cinerea* mycelium, strain 916T, after 7 days at 20°C.

The mycelium is cultured on a malt/glucose medium containing different concentrations of resveratrol.

Figure 10: Photographic plate 1

Macroscopic observations which are characteristic of different varieties of grapevine in interaction with *Botrytis cinerea* 5 days after inoculating vitroplant leaves with a conidial suspension - untransformed plants.

Upper row:

- Left: Folle blanche variety, clone 280, susceptible.
- Right: Pinot noir variety, clone 386, moderately tolerant.

Lower row:

- Left: Ugni-blanc variety, clone 479, tolerant.
- Right: 41B stock-vine, tolerant.

Figure 11: Photographic plate 2

Macroscopic observations which are characteristic of 41B stock-vine clones which have been transformed with different constructs (145: Ms PR10-1 promoter - *vst1* gene; 55: 13kb insert comprising two genes, *vst1* and *vst2*, under the control of their own promoters), in interaction with *Botrytis cinerea* 5 days after inoculating vitroplant leaves with a conidial suspension.

Upper row:

- Left: Clone 145-2.
- Right: Clone 145-5.

Lower row:

- Left: Clone 145-6.
- Right: Clone 55-3.

Figure 12: Photographic plate 3

Fluorescence microscopy observations which are characteristic of different grapevine varieties in interaction with *Botrytis cinerea* 5 days after inoculating vitroplant leaves with a conidial suspension.

Fluorescence: filter unit A (excitation of from 340 to 380 nm; stop filter at 425 nm). Resveratrol gives out a bluish white and blue (depending on its concentration) fluorescence light while chlorophyll fluoresces red. The black region corresponds to the region of fungal infection or to necrotic regions when they are small.

Upper row:

- Left: Folle blanche variety: clone 280, susceptible, little or no synthesis of resveratrol.
- Right: Pinot noir variety, clone 386, moderately tolerant. Synthesis of resveratrol in the veins and in the region of fungal maceration.

Lower row:

- Left: Untransformed stock-vine 41B (tolerant). Synthesis of resveratrol intense in the veins and in the lamina around the small and very localized regions of infection (necrotic regions).
- Right: Stock-vine 41B transformed with construct 145, i.e. the *vst1* gene promoter PMs PR10-1, clone 145-5, very tolerant. Strong synthesis of resveratrol around and in the region of infection, in veins and over almost the whole of the lamina of the leaf.

Example 1

Obtaining genomic clones which comprise regulatory sequences of lucerne PR protein genes

A) Obtaining a probe for the purpose of finding the promoters (cf. Figures 1 and 1a)

The incompatibility response (hypersensitivity reaction, HR) which is obtained in the lucerne (*Medicago sativa*) and *Pseudomonas syringae* pv *pisi* host/parasite relationship made it possible to construct a cDNA library. It was prepared from messenger RNAs which were extracted and purified from the zone adjacent to the necrosis caused by the bacterial infection. Plant material was taken 6 hours after infiltrating with the bacterial suspension.

The PR proteins in leguminous plants are known to have motifs which are conserved; it was therefore possible to synthesize oligonucleotides corresponding to these motifs defined on the basis of sequencing which had already been carried out on pea and soya bean PR proteins. PCR amplification enabled a radioactive probe to be obtained which was then used to select transcripts in the cDNA library. One of these clones, cDNA-PR7, was adopted since after sequencing it exhibited 87% homology with the genes encoding the pea and soya bean PR proteins. Analysis showed that it in fact corresponded to a gene encoding a class 10 PR protein according to the VAN LOON et al. (1994) classification. It was designated Ms PR10-1 (*Medicago sativa* PR class 10 protein, clone 1).

A control which was carried out on lucerne by means of Northern blotting showed that, in the incompatibility reaction, the corresponding transcript began to accumulate 3 hours after infection, passed through a maximum between 24 and 48 hours and decreased slowly from 72 hours onwards.

This fragment is characterized by the existence of an internal *Bam HI* site (marked with a B in Figure 2) which delimits two moieties:

- the one, termed 5' and of approximately 340 bases, includes the region upstream of the ATG (which is transcribed but not translated) and a downstream sequence corresponding to 306 bases,
- the other, termed 3', corresponds to the end of the coding moiety, i.e. 165 bases, and to the untranslated 3' region, that is 186 nucleotides from the stop codon to the beginning of the poly A.

B) Isolation of genomic clones comprising promoters of PR proteins

1) Isolation of genomic clones

A lucerne genomic library, prepared in EMBL4 (titre: 7.10^8 p.f.u. (plate-forming units) x ml^{-1}), was used on this occasion and 6.10^5 p.f.u. were plated out. The 5' fragment of Ms PR10-1 (cDNA PR7) was used as a probe to screen this library and 45 clones gave a signal, which was strong for 13 of the clones. Restriction mapping and hybridization which were carried out using the 5' and 3' fragments of Ms PR10-1 led to the conclusion that there were 7 distinct clones (cf. Figure 2). Comparison of the sizes of the 7 clones (9.3; 6.5; 6.1; 5.8; 4.8; 4.2; 2.2 kbp) which were obtained by screening the library with those of bands detected on a blot of lucerne genomic DNA showed good agreement between these two types of experimental data (cf. Figure 2). It was therefore possible to deduce from this that the genes encoding the PR7 protein corresponded to a small multigenic family.

The fragments (*EcoRI/EcoRI* sites) of these clones (apart from clone C12) were then subcloned in whole or in part and sequencing was undertaken.

2) Sequencing carried out

One clone was chosen for sequencing first, i.e. clone C15 (cf. Figures 1 and 1a).

The initial sequencing work demonstrated the presence of an intron of approximately 315 nucleotides in the open reading frame of the gene encoding the PR

protein. The clone under study was then analysed after having been digested with *Bam HI* (cf. Figure 1a).

Clone C15: 6.1 kb

Analysis of the clone with *Eco RI* and *Bam HI* enabled two fragments to be obtained, i.e. E-B (approximately 2.4 kbp) and B-E (approximately 3.7 kbp). After sequencing and comparing the coding sequence (interrupted by an intron of 600 nucleotides) with that of Ms PR10-1 (cDNA-PR7), it appeared that this genomic clone was absolutely identical to this reference cDNA.

3) Analysis of the expression of the isolated clones in the lucerne/*Pseudomonas* system

The experiments focused on clone C15, using the 5' extension technique in order to determine the messenger molecules which were in fact transcribed during induction of the defence reactions. This technique has the additional advantage of making it possible to locate the transcription initiation site. The results demonstrated that clone C15 was in fact expressed during induction of the defence reactions in the leaves as part of the lucerne/*Pseudomonas* interaction.

Example 2

Genetic transformations which were carried out in order to verify the promoter activity of the isolated clones

A) Promoter regions used

Two promoters were used for these verificatory transformations: a control promoter and the PR promoter which was isolated from the lucerne genome and which was derived from C15 (corresponding to the PMs PR10-1 promoter).

1) CaMV-35S promoter

This promoter, which is constitutive, is used as a standard promoter. It corresponds to the sequence for regulating the transcription of the gene for the 35S RNA subunit of the cauliflower mosaic virus (CaMV). The

promoter region which was used to effect the construct with the *gus* reporter gene in fact corresponds to a fraction of this promoter, which fraction was reisolated in the form of an *EcoRI/BamHI* fragment from the plasmid pDH51 (PETRZAK et al., 1986).

2) PR promoter

A study was carried out of the promoter regions of the C15 genomic clone. This promoter was subsequently termed PMs PR10-1.

PMs PR10-1:

It is derived from the *EcoRI/BamHI* (E/B) fragment of 2.4 kb of the C15 clone (Figure 1a). The integration of this fragment into the binary plasmid upstream of the reporter gene (see below) was made difficult by the fact that there was no restriction site in clone C15 between the TATA box (initiation of transcription) and the ATG (initiation of translation). Deletion experiments were therefore carried out until a fragment of approximately 1.5 kb was obtained (sequence IND S1). A *Bam HI* site was then added, by blunt-end ligation, to the resulting fragment in order to enable it to be inserted upstream of the different coding sequences which were subsequently used. This fragment therefore comprises, expressed by reference to the cDNA which was used to clone it and also the upstream promoter region: 39 terminal nucleotides of the 5'UTR (UnTranslated Region) of the Ms PR 10-1 gene, located 10 bp from the initiating ATG codon, the ATG of the Ms PR10-1 gene and a short fragment of its coding region (10 bp) immediately upstream of the integrated *Bam HI* site. Taking into account the cloning sites, the promoter which is constructed in this way has a potential ATG, which could lead to the presence of two ATG codons at a short distance from each other when constructing chimeric genes. There could then be a risk of altering the coding frame of the gene which is used (reporter gene or stilbene synthase gene).

For the transformation experiments with the

reporter gene, PMs-PR10-1 (PRI) was used as such after having been cloned into the STRATAGENE Bluescript pSK+/- plasmid. It was possible to reisolate it in the form of an approximately 1.5 kb *EcoRI/BamHI* fragment.

3) Plasmids used

a) p35S - *gus* intron (VANCANNEYT et al. 1990)

This plasmid is a derivative of pBin19 (BEVAN, 1984) and as such possesses the right and left borders of the binary plasmids, enabling the segment contained between these borders to be inserted into plants using agrobacteria.

The development of the *gus* intron (intron derived from the potato *LSI* gene) reporter gene made it possible to eliminate the false positives (in particular during transient expression) which were due to contaminating agrobacteria. These bacteria are not able to splice the introns.

In the standard system, this gene, which encodes a β-glucuronidase, enables a blue coloration to be obtained when a specific substrate (5-bromo-4-chloro-3-indolyl-β-glucuronide) is used. This blue colour then indicates that the analysed plant has been transformed and, as a consequence, that the coding sequence of the gene corresponding to the enzyme has been transcribed and, therefore, that the promoter which controls it has been induced.

b) pFR97

This plasmid was constructed by one of the laboratories participating in the project for testing promoter efficiency (P. RATET, ISV, cited in SZABADOS et al., 1995). It has, in particular, the advantage of possessing a multiple cloning site, making it possible to effect transcriptional fusion with the coding frame of the *gus* gene (E. coli *uid A*) containing the *LSI* intron. It also possesses some of the characteristics of the preceding plasmid p 35S *gus* intron (borders for integration into the plant genome, selection gene affording resistance to antibiotics of the neomycin

type: *npt II* gene).

The activity of the promoter can be visualized and measured by histochemical and enzymic tests of the GUS type.

4) Derivatives of pPR97

Two main constructs were made and subsequently used for transforming model plants.

a) pR97 - 35S

The 35S promoter, cloned into plasmid pDH51 (cf. paragraph 5 below), was excised and inserted into the multiple cloning site of pPR97, upstream of the reporter gene, in the form of an *EcoRI/BamHI* fragment. This plasmid is both a positive control, to demonstrate that the construct functions, and a reference since the 35S promoter was placed in the same environment as the promoter isolated from the PR protein clones.

b) pPR97-PMs PR10-1

The 1.5 kb fragment was inserted into the same cloning site as that defined above, in this case too in the form of an *EcoRI/BamH1* fragment.

c) pG3-3

This enabled a strong positive control for the histochemical and enzymic tests to be obtained by cloning two 35S promoters as an inverted tandem. The activator sequences of the promoters then act synergistically. The coding frame of the *gus* intron gene was then placed under the control of one of the two 35S promoters.

5) Preparation of Agrobacterium strains

The different plasmids were used to transform competent E. coli strain DH5α bacteria by thermal shock in a calcium chloride medium. After selecting transformed bacteria on a medium containing antibiotic (kanamycin) and using a miniprep to check that they were recombinant, these bacteria were then used to transfer the recombinant plasmids into Agrobacterium strains by means of triparental conjugation using the E. coli strain HB101 harbouring the autotransferable plasmid

pRK2013 (DITTA et al., 1985).

Two agrobacterium strains were used: EHA 105, a disarmed *Agrobacterium tumefaciens*, used for regenerating transformed plants (stable transformations) and A4TC24 *Agrobacterium rhizogenes*, which was used to obtain the hairy root reaction and composite plants whose roots are transformed but which otherwise have a phenotype which is identical to the original phenotype.

6) Genetic transformations on model plants

Two types of transformation (transient and stable) were carried out using three model plants, i.e. *Nicotiana benthamiana*, *Medicago truncatula* and *Lotus corniculatus*.

The results presented will in the main be those obtained with *N. benthamiana*.

7) Transient transformations

This first series of experiments was carried out for the purpose of rapidly verifying that the constructs which were produced using the *gus* gene functioned in the eukaryotic cells.

N. benthamiana leaves were therefore excised and cocultured on an agar medium together with the different derivatives of the EHA 105 strain. Histochemical tests were then performed 48 h after the transformation and subsequently examined after incubating overnight (12 h).

The control plasmids, p35S *gus* intron and pPR97-35S, gave a positive GUS coloration even if this was weak in the case of the second plasmid.

This weak reactivity is without doubt due to a construction problem since a part of the polylinker had to be retained in the vicinity of the transcription initiation site and the ATG of the *gus* gene. Since this polylinker part encompasses a repeat sequence it can interfere with transcription of the gene.

The pPR97-PMs PR10-1 construct gave a *gus* gene activity which was similar to that of the 35S *gus* intron positive control. This promoter, which was expected to

be inducible, therefore exhibited an effect which was comparable to that of a constitutive promoter.

This result can be explained as being the consequence either of the bacterial infection or the injuries inflicted on the leaves during sampling or during culturing. Since the first hypothesis cannot be verified, the experimental protocol was modified in order to decrease the stress caused to the explants (increase in the osmolarity of the coculture medium using sucrose concentrations of from 10 to 30 $g.l^{-1}$, applying a relatively high vacuum range: a relative vacuum of from 10 to 80 mm of mercury and the production of relatively severe lesions on the leaves accompanied by significant or moderate crushing of the epidermis).

The results demonstrated that the number of transformed cells increased as the pressure increased. However, a compromise should be found in order to achieve stable transformations since the large number of transient transformations which are obtained in this case frequently turn out subsequently to be lethal for the cells. The latter are then unable to give rise to cali and thus regenerate shoots and then plants. In any case, the inducible nature of the promoter is confirmed in part since, while the coloration due to the 35S-*gus* intron construct can be detected up to 5 days after coculturing, that obtained using pPR97 - PMs PR10-1-*gus* intron appears more rapidly at 48 hours but then subsides very rapidly.

8) Stable transformations

a) Tobacco: N. benthamiana

A transformation series was carried out using pPR97-35S, pPR97-PMs PR10-1 and pG3-3. A substantial number of plantlets was obtained for this transformation series. An attempt was made to obtain at least 7 acclimatized plants for each of the plasmids used. However, this was not possible for p35*S-gus* intron, where only 5 plants were regenerated and acclimatized. The results which were obtained are compiled in Table 1.

Table 1: Stable transformations obtained in *N. benthamiana* by transforming with the *Agrobacterium tumefaciens* strain EH 105 and its derivatives.

Constructs	Cali/explants cultured	Shoots obtained	Plantlets in vivo Accl.	
p35S-*gus* intron	10(45)	6(1)	5	5
pPR97-35S *gus* intron	115/122	23	13	12
pPR97-PMs PR10-1 *gus* intron	135/139	27	20	7
pG3-3-35S as an inverted tandem (strong promot.)	not assessed	37	28	20

Legend to Table 1:

The results are expressed as the quantity obtained.

Accl.: Acclimatized plantlets

Number of shoots per explant: The figure in brackets corresponds to the number of shoots which were obtained at one month in the case of the first series. In this series (comprising the 35S *gus* intron construct), the cali were left longer on the culture medium in order to obtain a maximum number of shoots and therefore of plants to acclimatize. In the case of the second series, a sufficient number of shoots had been obtained after one month and the experiment was then stopped.

Genetic transformations: These were done in a standard manner on 1 cm^2 pieces of leaf lamina, which pieces were immersed in the *Agrobacterium* suspension for 30 seconds and then cocultured for 48 hours before being subcultured onto agar medium for cell division and caulogenesis (MURASHIGE et al., 1962), 0.1 $mg.l^{-1}$ NAA (naphthaleneacetic acid), 1 $mg.l^{-1}$ BAP (benzylaminopurine), 400 $mg.l^{-1}$ cefotaxime (elimination of the agrobacteria) and 70 $mg.l^{-1}$ kanamycin (agent for selecting transformed cells). Once the first shoots have

appeared (approximately one month after coculturing), they are placed on a rooting medium which is identical to the former medium except that it does not contain plant hormones.

Rapid analysis of the results in terms of the expression of the *gus* intron gene depending on the nature of the promoter which is situated upstream of the coding frame of the gene shows that the PMs PR10-1 promoter gives the best results of all the promoters tested. A more detailed analysis is presented below.

9) Characteristics of the PMs PR10-1 promoter

Plasmid pPR97-PMs PR10-1-gus intron

This promoter gave the best results with differences in the constitutive expression of the *gus* gene depending on the organs tested.

a) Activity in cali

Strong constitutive expression was found. A few minutes of incubation were sufficient to obtain a positive histochemical test. The promoter is therefore strongly induced in this type of material, something which is in agreement with the results obtained by VAN LOON (1985). Cali which are cultured in vitro are in a state of stress and the PR proteins are expressed under these conditions.

b) Activity in acclimatized whole plants

Roots

The promoter is induced and the histochemical test is positive after 2 hours of incubation (as against 5 hours when the 35S promoter is used). The activity of the *gus* gene is not uniform in tobacco roots; only the epidermis of the old parts and the apical meristem gave the blue coloration which is characteristic for the test. According to the literature, defence gene activity in the roots is also observed under conventional conditions.

Flowers

A strong constitutive activity was found in the

flowers and, more particularly, in the anthers and the pollen, of all the tobacco plants which were transformed with this construct. *Gus* gene activity was also detected in the trichomes of the sepals and, more weakly, in the petals. These results are also in agreement with those of VAN LOON (1985), which indicate that defence genes are induced in the floral parts.

Leaves

Weak constitutive gus activity was observed in the trichomes of young leaves of adult plants. In the case of tobacco, the rosette stage with large leaves corresponds to the juvenile stage and the ageing stage to that of the formation of seeds. This weak activity was predominantly observed in the multicellular trichomes. To our knowledge, the expression of a PR protein in such structures has never been described.

While it is possible, therefore, that the isolated PMs PR10-1 promoter has a constitutive inducing activity in the trichomes of tobacco leaves (3 plants out of 7), this activity appears to be under the influence of the development stages.

In the absence of induction by a pathogen, the activity of the promoter in tobacco is therefore limited to the root, to floral parts (anthers and pollen) and to a few cells of the aerial part (basically trichomes).

Example 3

Other plant species which were transformed

1) Medicago trunculata

In the case of this species, an attempt was made to create composite plants, that is plants which possess, at one and the same time, a wild-type aerial part (not genetically transformed) and transformed roots.

Young germinations were used. After development of the main root, the excised hypocotyles were soaked in a suspension of *Agrobacterium tumefaciens* EHA 105 harbouring either plasmid p35S-*gus* intron or plasmid

pPR97-PMs PR10-1-*gus* intron so as subsequently to obtain newly formed roots which were transformed. After one week, roots were obtained and a histochemical GUS test was carried out. For this experiment, the control consisted of young germinations which were treated in an identical manner to the previous batches (hypocotyles were excised, but were not soaked in the agrobacterial suspension).

While all the explants of the control batch formed new roots within one week, only 50% of the explants reacted, by contrast, in the case of the batches which were treated with the agrobacteria. Whatever the treatment, no root gave a positive response to the GUS test. On the other hand, even though necrosed, the base of the hypocotyles in the treated batches often reacted by giving a blue coloration (presence of tranformed cells). The necrosed part of the explants was then excised and they were set to rooting again. 50% then developed newly formed roots, some of which were to be positive to the test in a few regions.

Chimeric roots (roots containing both transformed cells and untransformed cells) were therefore obtained, with the transformed parts corresponding to cell lines which had integrated the construct into a basal stem cell.

The two constructs which were tested, i.e. p35S-gus intron and pPR97-PMs PR10-1 *gus* intron, gave these transformed root cell lines in 3 and 2 explants, respectively, out of the 6 which were subjected to experiment in the case of each batch.

Although the experimental model was not adapted to the topic under investigation (study of the expression of PR proteins in association with the phenomenon of nodulation by *Rhizobium*), it nevertheless demonstrated that the PMs PR10-1 promoter is just as functional in this plant as in the original plant (*Medicago sativa*).

2) Lotus corniculatus

In this case too, the experiment had the aim of studying the induction of the promoter in association with nodulation by the symbiotic bacterium *Rhizobium meliloti* NZP 2037 (PETIT et al., 1987). Composite plants were therefore produced by transforming cells of the hypocotyl of young Lotier germinations with *Agrobacterium rhizogenes* strain A4TC24 in order to obtain the hairy root phenomenon (hairy root phenotype). Once this had developed, the main roots were excised and the plantlets were placed in a liquid medium in order to amplify the development of the phenomenon. Once the plants were acclimatized, induction of the promoters in association with nitrogen fixation symbiosis was studied by putting the plantlets under nodulation conditions (BLONDON, 1964). The two promoters which were used for the study were the same as those which were used in the experiment carried out with *M. trunculata*: 35S and PMs PR10-1. Using these two constructs, less than 10% of the roots having a hairy root phenotype exhibited roots which were positive to the GUS test. Generally speaking, the roots having this phenotype gave fewer nodules than did the control roots.

In the case of those which were obtained with the construct comprising the 35S promoter, it was only the nodules which gave a positive response to the GUS test, while, in the case of the other construct (PMs PR10-1 promoter), the coloration developed over the whole of the root apart from the secondary root initiation point. Apart from that, this latter construct did not enable nodules to be obtained on the roots derived from hairy root in interaction with *Rhizobium meliloti*.

Example 4

Study of the hypersensitivity reaction of tobacco which is transformed with the constructs using promoters of the lucerne PR gene and the gus intron gene

A) Hypersensitivity reaction in *N. benthamiana* which is transformed with the constructs linking the lucerne PR gene promoter and the *gus* intron gene

1) Hypersensitivity reaction (HR) test

The hypersensitivity reaction (HR), developed in the *N. benthamiana/Pseudomonas syringae* pv. *pisi* interaction, was used in this study. Transformed tobacco plants, which had incorporated the different inserts of plasmids p35S *gus* intron and pPR97- PMs PR10-1-*gus* intron into their genome, were acclimatized and then infiltrated with a suspension of *P. syringae* (ESNAULT et al., 1993) at a concentration of 10^9 bacteria per ml. The solution was injected into the lamina using a hypodermic syringe. Using a model of this nature, the HR reaction is regarded as being well developed after 48 hours. Leaves which had been infiltrated with the bacterial suspensions were removed at 24, 48 and 96 hours after inoculation in order to assess induction of the different promoters under study using the GUS histochemical test. The same histochemical test was also used on the leaves situated below the infiltrated leaf in order to assess any possible systemic response.

2) Study of the induction of the promoters under the HR reaction conditions

a) 35S constitutive promoter

In the case of the 35S constitutive promoter (plasmid pG3-3, for example), the inoculation with *P. syringae* did not modify the response to the test, with this response being evident after a few minutes of incubation. Therefore, infiltration with the bacteria does not alter the constitutive glucuronidase activity which is obtained with the 35S promoter.

b) Promoters of PR protein genes: Ms PR10-1 promoter

As shown in Table 2, this promoter is readily inducible by pathogen attack. While the HR reaction is still not completely developed at 24 hours (48 hours for the effect to be fully displayed), the GUS test is already positive. In the case of the young transformed tobacco plants which were obtained, the coloration is weak and predominantly produced in the lamina of the infiltrated leaf.

With regard to the systemic response, i.e. the response in the leaf below the infected leaf, the coloration is only present in the lamina. The adult (having developed stems but still not having flowered) and juvenile (rosetted) tobacco plants have the same type of response, with a weak *gus* gene activity as determined by the histochemical test.

In the case of the older tobacco plants, either in flower or carrying seeds, the coloration which is obtained in the test is more intense, especially in the veins and the trichomes of the infected leaf, with coloration only being present in these tissues in the case of the systemic response.

These observations therefore demonstrate differences in the expression of the reporter gene depending on the age of the plant. This response, which is dependent on the developmental stage of the plant, was found in most of the studies carried out on plant PR proteins. Induction of the PMs PR10-1 promoter is a transient phenomenon since expression of the reporter gene is no longer evident at 96 hours after inoculating the bacteria.

The induction is not limited, either, to the HR reaction which is obtained in the plant/bacterium interaction. The same type of response was obtained with the PMs PR10-1-*gus* intron construct when one of the explants was infected with a fungus. Homogeneous expression of the *gus* gene was then evident over the entire infected leaf apart from the contaminated region, which was necrosed.

Table 2: Induction of the different promoters under study in association with the HR reaction between *N. benthamiana* and *P. syringae*.

Construct	24 h after inoculation		96 h after inoculation
	Infiltrated leaf	Leaf below	Infiltrated leaf HR reaction
PMs PR10-1	6/7	6/7	0/3
pG3-3 (35S)	3/3	3/3	2/2

Legend to Table 2:

The results are shown as the number of plants responding positively to the GUS histochemical test as compared with the number of plants which were analysed.

B) Quantitative expression of the *gus* intron gene under the control of the different promoters

The method is based on an enzyme test.

The method employs:

a) a crude extract of the enzyme encoded by the *gus* gene, with the enzyme being obtained from transformed tobacco plants, and

b) a substrate, i.e. p-nitrophenyl-glucuronide. The rate at which the substrate is hydrolysed is monitored in a spectrophotometer and is related to the total quantity of protein in the extract. On the other hand, since it is not very sensitive, the method requires the presence of a strong promoter upstream of the *gus* gene.

As a consequence, it was not used for assays which required 12 hours or more of incubation with the substrate which was used for the histochemical test (X gluc: 5-bromo-4-chloro-3-indolyl-β-glucuronide).

Under these experimental conditions, the 35S promoter, located in plasmid pG3-3, gave a rate of substrate hydrolysis (expressed in arbitrary units) which was 5 times greater than that achieved using the

PMs PR10-1 promoter, which was located in plasmid pPR97. On the other hand, the PMs PR10-1 promoter gave a stronger expression of the *gus* gene than did the 35S promoter when the latter was located in plasmid p35S-*gus* intron. In fact, no substrate hydrolysis was detected in this latter case.

Similarly, it was not possible to obtain spectrophotometrically detectable values when the other constructs were used.

Example 5

Isolation of the DNA corresponding to a stilbene synthase gene and expression of stilbene synthase

Two methods were used for obtaining a gene encoding grapevine stilbene synthase. On the one hand, data in the literature (WIESE et al., 1994) allowed the sequence of a gene to be obtained. On the other hand, a genomic insert of approximately 13 kb was supplied by BAYER AG (Agrochemical Division Research/Biotechnology-Pflanzenschutzzentrum, MONHEIM, D-51368 LEVERKUSSEN). It is specified that the company BAYER has deposited with the Deutsche Sammlung von Mikroorganismen (DSM), in Germany, strains of *E. coli* containing plasmids bearing grapevine stilbene synthase genes (cf. EP-464 461): *E. coli* strain Fier 1 pVst 1 (DSM 6000, deposited on 18 June 1990), *E. coli* strain Fier 2 pVst 2 (DSM 6003), deposited on 18 June 1990) and *E. coli* strain Fier pVst 1 2 to 3 (DSM 6346, deposited on 11 February 1991). BAYER has also deposited the *E. coli* strain Nurdug 2010 (DSM 4243, deposited on 17 September 1997) which contains the plasmid pGS 828.1 which bears a groundnut stilbene synthase gene (cf. EP-309 862). The insert used for carrying out the work reported below in fact corresponds to a complex genomic clone which comprises two complete functional stilbene synthase gene sequences (*vst1* and *vst2* genes) and an incomplete *vst3* sequence. Subsequently, the sequence of the *vst1* gene was chosen for incorporation into the constructs produced. It corresponds to a 4.9 kb genomic fragment (functional

sequence including the promoter) which does not possess any restriction sites which are suitable for cloning it directly into the plasmids which are usually used as transformation vectors. Additional sites were therefore added to it by carrying out an intermediate cloning in a plasmid termed pCDNA II.

A) Addition of supplementary sites by intermediate cloning in a plasmid pCDNA II

The abovementioned genomic fragment of the *vst1* gene was isolated from the original plasmid in which it had been cloned in the form of an *EcoRI/PstI* fragment (2.1 kb) and cloned again into a plasmid pUC19. Having been cloned, the *vst1* fragment was incorporated into the same sites (*EcoRI/PstI*) of plasmid pCDNA II in order to change the restriction sites, delete the terminator of the gene and enable a *BamHI/BamHI* (1.8 kb) insert to be isolated. It was this fragment, corresponding to the open reading frame of the gene, which was used for making the constructs with the different promoters including those isolated from the lucerne genomic library (cf. Figure 4). This insert was then cloned into pBIN 19 after using Southern blotting to verify the sizes of the different fragments which were obtained after digestion with appropriate restriction enzymes.

B) Study of the expression of the *vst1* gene

In order to verify the expression of the genes encoding stilbene synthase in the grapevine plants, a probe (1.8 kb) encompassing the *vst1* gene was prepared from plasmid pCDNA II, which had been multiplied in the bacterium *E. Coli* HB 101. The probe was then biotinylated by random priming, using the Polar Plex kit (Plex chemiluminescent kits, Millipore) in order to enable genes or transcripts encoding a stilbene synthase to be detected by chemiluminescence on Southern and Northern blots carried out using nucleic acids which were extracted from control or transformed grapevine plants.

1) Use of the *vst1* probe for analysing genomic DNA extracted from grapevine 41B (*V. vinifera Chasselas x V. berlandieri* hybrid; stock-vine)

Southern blot analyses were carried out after extracting genomic DNA and then digesting it with *EcoRI*. Use of the *vst1* probe enabled a large number of bands (approximately 15) to be obtained. These bands in fact correspond to fragments which contain sequences encoding a stilbene synthase and which constitute a multigenic family (from 6 to 8 genes according to WIESE et al., 1994). Of these, *vst1*, *vst2* and *vst3* exhibit strong homology with each other. Furthermore, other genes can be recognized by this probe, in particular those corresponding to the chalcone synthetase multigenic family. This is because the two enzymes have the same structure and are of the same size (dimers of subunits of from 41 to 44 kb in size). They also use the same substrate and their amino acid sequences exhibit a high degree of homology, at least with regard to the active site.

The DNA of two plasmids was extracted for the purpose of verifying whether such a cross-hybridization was possible. One of the plasmids, pCDNA II, contained the *vst1* gene while the other, pPCV 002, contained a Rosier chalcone synthase gene, available in the laboratory. A biotinylated probe corresponding to the Rosier chalcone synthase gene was also prepared. The Southern blots obtained by carrying out cross-hybridization showed that the *vst1* probe actually recognized the Rosier chalcone synthase fragment, and vice versa. The emitted signals are then weaker in this case of cross-hybridization.

2) Assay of resveratrol using grapevine plant leaves

The fresh plant material, leaves or stalks, is reduced to powder in a mortar containing liquid nitrogen, and the apolar compounds are extracted with methanol (1 ml per 100 mg of fresh material : f.m.).

After centrifugation to remove debris, the methanol extract is filtered through a 0.45 µm filter and then evaporated to dryness under nitrogen. The residue is taken up in pure methanol (100 µl/100 mg f.m.). In order to remove pigments (in particular chlorophylls), the sample is then passed over a C18 column (Sep-pack WATERS), which has been pre-equilibrated with methanol. The qualitative and quantitative analysis of the extracts is carried out by H.P.L.C. (High Pressure Liquid Chromatography) on a WATERS H.P.L.C. (Model 600 E) coupled to a diode-array detector (Model 990. WATERS). The chromatography support is composed of a reverse phase C18 column (C18 ultra base, 205 × 4.6 mm, 5 µm; Shandon). The analysis of the extract compounds is carried out under isocratic conditions, the mobile phase consisting of a 35/65, V/V, acetonitrile/water mixture with a flow rate of 1 ml × min^{-1}.

An absorption spectrum is performed every two seconds between 200 and 400 nm, and the resveratrol is detected at its adsorption maximum at 305 nm.

The quantification of the resveratrol is carried out by external calibration using a standard straight line obtained by chromatography of solutions at 5, 10, 20, 50 and 100 µg · ml^{-1}, carried out with commercial resveratrol (Sigma). The resveratrol concentration, which is measured from the area under the peak corresponding to the molecule, is related to the unit mass of f.m. or of d.m. (dry matter) or chlorophyll mass of the sample assayed.

Example 6

Nucleic acid constructs which link the gene encoding a grapevine stilbene synthase to different promoters

1) Constructs using constitutive promoters

Two related promoters were used for making the genetic constructs permitting constitutive expression.

In the first construct, the DNA of grapevine stilbene synthase (*vst1*) was placed under the control of

a regulatory sequence which consisted of a cassette containing two CaMV 35S promoters arranged in series (in the same orientation). A 35S polyadenylation sequence was also added to the end of the sequence encoding the *vst1* gene. The chimeric construct which was made in this way can therefore be summarized as follows:

(CaMV) p35S - (CaMV) p35S - *vst1* - (CaMV) 35S poly A

In the second construct, the *vst1* coding sequence was placed under the control of four enhancer sequences, which sequences were isolated from the CaMV 35S promoter and arranged in series upstream of the CaMV 35S promoter and the native enhancer sequence of the promoter of the grapevine gene (*vst1*). The two chimeric sequences which were produced in this way were first of all inserted into the plasmid PMP 90RK and the plasmids were then subsequently incorporated into the Agrobacterium strain GV3101.

These two regulatory sequences are regarded as being "strong" constitutive promoters.

2) Homologous construct using the 13 kb insert (*vst* genes under the control of their native promoters)

The grapevine genomic DNA fragment of approximately 13 kb in size was described above. It encompasses, in particular, two functional genes (*vst1* and *vst2*) encoding the stilbene synthase enzymes and one incomplete (non-functional) gene (*vst3*). This grapevine sequence was cointegrated into a plasmid pGV3850, which was then introduced into an Agrobacterium strain. The sequence therefore corresponds to the open reading frames of *vst* (grapevine stilbene synthase) genes under the control of their native promoters.

Several series of 41B plants, which were transformed with the plasmid containing the 13 kb fragment, were obtained and were studied and analysed by Southern blotting using three different probes (1.8 kb probe from the *nptII* gene; 2.4 kb probe from the gene for resistance to *ampicillin* and 1 kb probe from the left-hand border of plasmid pBIN 19, comprising the final

integration sequence of the TDNA). The majority of the plants which were used reacted to one or other of these probes. These clones were numbered according to a code: 55 for the construct and 2, 3, 5, 6, 7 and 9 for the different transformants which were obtained.

3) Constructs using the inducible PMs PR-10-1 promoter

The construct containing the PMs PR-10-1 promoter, which was isolated from lucerne genomic PR clones, was made (cf. Figure 5).

Construction of the plasmid pBin 19 - PMs PR-10-1 - *vst1*-35S terminator gene

Since the PMs PR10-1 promoter which is isolated from lucerne and the *vst1* gene each contain a translation-initiating ATG codon, an adaptor was made in order to clone the *vst1* gene into the construct without an additional ATG. This adaptor was synthesized in the form of two oligonucleotides of 11 bp in each case, one of which was *Bam HI*-compatible while the other was *Mun I*-compatible. The adaptor was then incorporated into the *Mun I* site of the *vst1* gene which was cloned into the plasmid pUC19. The insert was then recovered by digesting with *Bam HI*, after which it was cloned into pBIN 19 between the PMs PR10-1 promoter and the 35S terminator. The insert of the *vst1* gene, together with its adaptor, is therefore located between the PMs PR10-1 promoter and the 35S terminator. Under these conditions, the ATG of the *vst1* gene was removed and 3 additional codons were included in the *vst1* coding frame upstream of the gene. It was checked by sequencing that the open reading frame of the gene was still in phase with the remainder of the construct.

After having transformed 41B plants with the insert containing, inter alia, the chimeric sequence: PMs PR10-1-promoter-*vst1* gene 35S terminator, the transformants were analysed by Southern blotting using the *nptII* probe which has already been described. Nevertheless, it is not fully possible to demonstrate

complete integration using this method and, in particular, this probe, since, in the *Agrobacterium* system, it is accepted that insertion into the genome of the plant begins at the right-hand border and terminates at the left-hand border. Since, in the construct which is used, the *nptII* gene is located close to the right-hand border, it is therefore possible for the integration to be partial, with the *nptII* gene being inserted but with there being a subsequent block in integration before the left-hand border is reached. The transformants were coded 145 and allocated the numbers 2, 5 and 6 for those transformants whose results are presented below.

Example 7

Genetic transformation of the grapevine and analysis of the efficiency of the promoters being studied

As described above (Example 6), four main constructs were produced for transforming the 41B stock-vine (*V.vinifera* × *V. berlandieri)* laboratory model system. The reason for using the latter system is that it has the advantage of giving good results in the transformation of embryogenic cell suspensions with agrobacteria. Approximately 50 transformants are obtained on average in experiments using from 0.1 to 1 µl P.C.V. (packed cell volume) of embryogenic cells. Furthermore, selection, development of the transformed embryos and regeneration into plants are all rapid. Plantlets having from 6 to 8 well-developed leaves can be obtained in vitro in two months of culture.

A) Genetic transformation of 41B with vectors containing the *vst1* gene under the control of constitutive promoters

Two constructs were tested, the one containing the 35S double promoter arranged in series upstream of the *vst1* gene and the second consisting of a regulatory sequence composed of 4 CaMV 35S enhancer sequences arranged in series upstream of the CaMV 35S promoter,

with the whole being located upstream of the *vst1* gene possessing its native enhancer sequence. Four trial series were performed (two for each of the constructs) with the aim of transforming the embryogenic grapevine cells. None enabled plants or even embryos to be regenerated. In every case, rapid necrosis of the embryogenic cell suspensions was obtained after the 48 hours of coculture with the agrobacteria. These constructs cannot be used to obtain transformed plants which are constitutively expressing the *vst1* gene under the control of these "strong" promoters. One hypothesis may be put forward; it is possible that expression of this gene blocks the regeneration into embryos due to the rapid necrosis of the potentially embryogenic cells in response to the production of stilbenic phytoalexins.

These negative results, which were obtained with the constructs containing constitutive promoters of 35S type, demonstrate the importance of controlling overexpression of the *vst1* gene by means of a homologous or heterologous promoter which can be induced, in particular, by a pathogen.

These present results are similar to those which were published by FISHER and HAIN in 1994 and which underline the fact that they did not succeed in constitutively expressing the groundnut stilbene synthase gene at an elevated level in tobacco when using the CaMV 35S promoter. According to these authors, when such a construction is used, expression of the gene would be regulated negatively when the plant is attacked by a pathogen. This regulation, according to their hypothesis, could result from the deployment, by the plant, of defence mechanisms which are normally induced by the pathogens (synthesis of PR proteins, in particular) and which would exert an inhibition on the viral promoters.

B) Genetic transformations of 41B with vectors containing the *vst1* gene under the control of promoters which can be induced by abiotic and/or biotic stresses

1) Study of the expression of the *vst* genes, and of their kinetics of induction with UV light, in excised leaves which are isolated in the surviving state, and in whole-plant control 41B grapevine vitroplants or 41B grapevine vitroplants or which are transformed with the 13 kb insert

It has been demonstrated that the synthesis of resveratrol (main grapevine phytoalexin), which is a product of the reaction catalysed by an enzyme stilbene synthase can be induced by ultraviolet (UV) light, that is under conditions of abiotic stress (LANGCAKE et al., 1977; SBAGHI et al., 1993). Under normal conditions, grapevine synthesizes little or no resveratrol; by contrast, following induction with U.V. light (10-minute exposure to UV light at 254 nm, for a lamp dissipating 600 $\mu W.cm^{-2}$), followed by a period of 20 hours in the dark, the phytoalexin is synthesized in the excised leaves.

a) Method used

Irradiation of the leaves:

- leaves of vitroplants: at 254 nm for a lamp dissipating 600 $\mu W.cm^{-2}$ vitroplants for 13 minutes,
- leaves isolated from vitroplants or at 254 nm for a lamp dissipating 600 $\mu W.cm^{-2}$ for 8 minutes,
- extraction and analysis of the mRNA from the plant material and estimate of the resveratrol by fluorescence after excitation at 365 nm at a given time after induction.

Each sample consists of 3 leaves which have been isolated from the same plant and which have been separately induced by UV light but then recombined for the extraction and assay. The test is performed on excised leaves, which have been isolated from vitroplants or on

vitroplants which are being cultured on agar medium and which possess from 6 to 7 well-developed leaves. The three oldest leaves on each plantlet are removed and used for the test. The upper surface of the leaves is exposed to the UV light. Following analysis, the quantities of resveratrol obtained are expressed in µg of product either per gram of fresh weight of analysed leaves or per gram of dry matter (estimated on the pellet after centrifuging and after extraction with methanol) or in mg of resveratrol per g of chlorophyll.

The tables given below show the values which were obtained for the 41B and 55-X clones, which were transformed with the 13 kb genomic clone construct in which two genes, i.e. vst1 and vst2, both of which genes are under the control of their native promoters, are present in the sequence used as insert.

b) Study of abiotic (UV light) stress on excised leaves (experiment No. 1)

The results, corresponding to construct 55 (13 kb insert) and to clones 2 (55-2) and 3 (55-3), are presented in Tables 3 and 4 below (assay of resveratrol) and depicted in Figures 6 and 7 (analysis of the transcripts). The study of the kinetics of the induction by UV light of the expression of the genes encoding stilbene synthase was performed after a period which separated the induction with UV light from the analyses and which was either fixed at 17 hours (cf. Table 3 and Figure 6) or was a variable period of 0, 8, 17, 24 or 32 hours after induction (cf. Table 4 and Figures 7 and 8).

Table 3: Quantities of resveratrol which are detected in uninduced control and transformed leaves or in control and transformed leaves at 17 hours after induction with UV light (expressed in $\mu g.g^{-1}$ of fresh material)

Untransformed controls		Clone 55-2		Clone 55-3	
Uninduced	Induced	Uninduced	Induced	Uninduced	Induced
0	12	0	11	0	13

Legend to Table 3: The leaves were excised from 41B vitroplants prior to induction with UV light. Clones 55-2 and 55-3 correspond to transformed plants which have integrated an insert of 13 kb which contains genes encoding stilbene synthase.

The fluorescence, which is observed in the blue/violet band at approximately 450 nm, is very strong in the veins of the excised leaves and is distributed uniformly over the entire surface of the leaf. The control leaves, which have not been induced with UV, do not exhibit any fluorescence. These results demonstrate specific expression of the genes encoding stilbene synthase. Analysis of the transcripts by means of Northern blotting, which was carried out using the vst1 probe, demonstrates the presence of a fragment of approximately 1.8 kb in size whose emitted signal is very intense in the case of the leaves induced with UV light but which is absent or very weak in the case of the uninduced plants (cf. Figure 6).

Table 4: Kinetics of the development of resveratrol concentrations in 41B vitroplant leaves which have been induced with UV light, removed from the plants being cultured on agar medium, and then isolated and analysed for their resveratrol content at various periods after induction.

Time after induction (h)	Quantity of resveratrol (in $\mu g.g^{-1}$ of fresh material)		
	Control	PCT 55-2	PCT 55-3
NI	0	0	0
0	0.6	0	0
8	30.8	12.1	9
17	56.8	71.7	47
24	83.2	81.4	35.2
32	15.5	7.7	151.8

Legend to Table 4: The results are expressed in $\mu g.g^{-1}$ of fresh material. Two plants, transformed with PCT 55-2 and PCT 55-3 respectively, were analysed in comparison with the control.

NI: Not induced with UV light.

Under these conditions, after stressing with UV light, the quantity of resveratrol found in the control leaves is at a maximum at 24 hours after induction (of the order of 80 $\mu g.g^{-1}$ of f.m. for 41B). However, there are significant variations depending on the date of the analysis in the cycle of subculturing the vitroplants: micropropagation cycle.

The results obtained after Northern blot analysis using the *vst1* probe are depicted in Figure 7. At least two types of transcripts, of very similar size (approximately 1.8 kb), were detected. Although cross-hybridization with a chalcone synthetase transcript cannot be excluded, the different messenger RNAs which were recognized probably correspond to the expression of different stilbene synthase genes. Thus, it has been shown in the grapevine (WIESE et al., 1994; KINDL, personal communication) that differences exist in the kinetics of induction of the different genes of the

multigenic family encoding stilbene synthases.

Northern blot analysis demonstrated that, in the leaves which were excised from grapevine 41B and then subjected to the abiotic stress which exposure to UV light represents, the stilbene synthase transcripts exhibit an expression maximum at approximately 17 hours to 32 hours after induction. These results are comparable with those obtained with grapevine cell cultures, which also demonstrated the same pattern of expression but with the presence of two maxima (WIESE et al., 1994).

c) Study of abiotic stress (UV light) in the isolated surviving leaf (experiment No. 2), induction in vitroplants before isolation of the leaves

Table 5 below presents the results which were obtained for a second series of transformants which had integrated the 13 kb insert. The study of the kinetics of the induction, with UV light, of the expression of the genes encoding stilbene synthase was performed after a variable period, separating the induction with UV light from the analyses, of 20, 40 or 60 hours.

Table 5: Concentration of resveratrol in the isolated leaves of grapevine vitroplants after induction with ultraviolet light and extraction at different survival times.

Clone studied	Survival time of the leaves isolated from vitroplants after induction with UV light (8 min at 254 nm) and before extraction of the resveratrol		
	20 hours	40 hours	60 hours
41B - control not induced	0	0	0
41B - control induced	281	165	6
Clone 55-2 induced	135	918	58
Clone 55-3 induced	44	931	301
Clone 55-5 induced	392	83	657
Clone 55-6 induced	339	235	43
Clone 55-7 induced	263	411	148
Clone 55-9 induced	386	823	54

Legend to Table 5:

The concentrations of resveratrol are expressed in $\mu g.g^{-1}$ of dry matter.

41B stock-vine hybrid *V. vinifera Chasselas* x *V. Berlandierri*.

The results presented in Table 5 enable two groups of plants to be distinguished:

- The first corresponds to the control and to two of the transformants, i.e. 55-6 and 55-7. In general, they exhibit a maximum resveratrol concentration of between 280 and 410 $\mu g.g^{-1}$ of dry material, with this maximum

generally being at 20 hours after induction, except for clone 55-7, when it is at 40 hours.

- The second group exhibits maxima which are higher, that is almost double those of the first group (from 820 to 930 $\mu g.g^{-1}$ of dry material). This group consists solely of transformants (55-2, 55-3 and 55-9). The maximum is expressed at 40 hours after the induction with UV light; on the other hand, at 20 hours after induction, these plants frequently have concentrations which are much lower than those of the first group. This is the case, for example, for clones 55-2 and 55-3 (135 and 44 $\mu g.g^{-1}$ of dry material, respectively). However, one of the clones, i.e. 55-9, is of interest since it exhibits resveratrol concentrations which are greater than those of the control in all cases (386, 823 and 54 $\mu g.g^{-1}$ of dry material at the times of 20, 40 and 60 hours after induction).

In this surviving leaf system, an uninduced control never exhibits resveratrol and, in almost all cases (apart from clone 55-5, which behaves in a distinctive manner), the resveratrol concentration falls drastically at 60 hours after induction.

d) Study of abiotic stress (UV light) which is carried out on vitroplants which are being cultured on agar medium

This study, which is carried out on a plant which is growing on an agar medium, makes it possible to analyse the production of resveratrol when other defence mechanisms of the plant may be being expressed, at least those mechanisms which are capable of being expressed under the in-vitro culture conditions. Furthermore, the study makes it possible to monitor the synthesis of resveratrol over a longer period (in the preceding studies, the isolated leaf necroses beyond 72 hours).

Four transformed 41B vitroplant clones in culture (55-2, 3, 5 and 6) were treated with UV light under the same conditions as before (8 min at 254 nm), with the leaves only being removed from the plant at

20 hours after induction. The results which were obtained were compared with the untransformed control as well as with a clone of *Vitis rupestris* and with 3 clones of *Vitis vinifera* variety which are known in the field for having a relatively high susceptibility to attacks by *Botrytis* on their berries.

The literature (SBAGHI et al., 1993) in fact demonstrates that a correlation exists between the susceptibility of the berries to *Botrytis*, as assessed in the vineyard, and the content of resveratrol in the leaves of vitroplants which had been induced with UV light. The results are presented in Table 6 below.

Table 6: Results of HPLC assays of resveratrol which were performed at 20 h after inducing several different varieties of grapevine with UV light of 254 mn for 8 min.

Variety tested	(resveratrol) $\mu g.g^{-1}$ of dry weight
Rupestris 215	351
Stock-vine 41B	237
Ugni-blanc 479	209
Pinot noir 386	86
Folle blanche 280	37
Pct 55-2	361
Pct 55-3	235
Pct 55-5	115
Pct 55-6	539

Legend to Table 6:

The induction with UV light is performed on vitroplants which are being cultured on agar medium. The leaves are removed and extracted for analysis at 20 hours after the treatment.

PCT 55-2, 3, 5 and 6 - transformants which have integrated the 13 kb insert into their genome. The results show the average of 3 repetitions.

These results provide a good indication that a correlation exists among the varieties and grapevine plants between susceptibility to *Botrytis* and content of resveratrol in the leaves at 20 hours after induction. Two groups can be identified in the untransformed varieties and grapevine plants. The first, which is formed by *V. ruspestris, V. vinifera x V. berlandieri* (41B) and Ugni-blanc, corresponds to those which are relatively tolerant to the fungus. The second, consisting of the Pinot noir and the Folle blanche, represents those which are regarded as being moderately tolerant to very susceptible (Folle blanche for example).

As far as the transformants are concerned, they have, on average, resveratrol contents at 20 hours after induction which are greater than or equal to the untransformed 41B control (539, 362 and 236 $\mu g.g^{-1}$ of dry material for clones 55-6, 55-2 and 55-3, respectively, and 237 for the control).

By contrast, the concentration obtained in the case of the transformed clone 55-5 is half that of the control. If these values, expressed in $\mu g.g^{-1}$ of dry weight, are compared with those of the isolated leaves (Table 5), it is seen that they are similar in the case of the control but that, on the other hand, variations exist in the case of the transformants which are analysed at 20 hours after induction. These variations are at times very substantial (135 for induction in isolated leaves and 362 for induction in vitroplants in the case of clone 55-2; 44 against 236 in the case of clone 55-3; 392 against 116 in the case of clone 55-5 and 339 against 540 in the case of 55-6). In addition, a great variability exists among the various repetitions.

2) Comparative study of the expression of the *vst* gene and of its kinetics of induction by biotic stress in 41B

grapevine vitroplants which have been transformed with the 13 kb insert and with the construct comprising only the *vst1* gene under the control of the PMs PR10-1.

The results above (chapter 1), which were obtained in vitroplants which had integrated, by genetic transformation, additional copies of stilbene synthase genes (in the form of a 13 kb insert comprising two functional *vst1* and *vst2* sequences), show that an overproduction of resveratrol, which is produced from the reaction catalysed by the stilbene synthase enzyme, is possible in some transformants when these genes are under the control of their own promoters, and in response to an abiotic stress such as U.V. irradiation.

The production of resveratrol was then verified with respect to these results, in two types of transformant, the first representing a 41B clone which had integrated the 13 kb insert (*vst1* and *vst2* stilbene synthase genes under the control of their own promoters), the second representing several clones which had incorporated only the *vst1* gene under the control of the lucerne defence gene regulatory sequence PMs-PR10-1. This comparison was carried out after induction by a biotic stress caused by *Botrytis cinerea*.

a) Methods used

α) Demonstration of the fungitoxic effect of the resveratrol molecule on *Botrytis cinerea*

The data in the literature with regard to the fungitoxic nature of the molecule are contradictory. According to DAÏ (1994), resveratrol does indeed exhibit an inhibitory effect on the development of the zoospores of *Plasmopora viticola* (agent of mildew); by contrast, PONT and PEZET (1990) maintain that it does not block germination of *Botrytis cinerea* conidia.

A study was carried out of the action of the molecule on the growth of mycelial hyphae of *Botrytis cinera* in culture on a malt/agar medium containing a resveratrol dilution range of from 10^{-1} M to $3.7.10^{-3}$ M,

with the hyphae being incubated at ambient temperature for 7 days. The results demonstrated that the molecule had an inhibitory effect (IC_{50} = 500 $\mu mol.l^{-1}$), with an exponential decrease in the mean growth diameter of the fungus for concentrations greater than 125 $\mu mol.l^{-1}$. On the other hand, concentrations of 37 $\mu mol.l^{-1}$ only have a very slight inhibitory activity on the growth of the mycelium (cf. Figure 9). However, this inhibition increases progressively after 7 days of culture under these conditions, which are otherwise very favourable to the growth of the fungus. After 20 days, the fungus finally infects the whole of the culture surface.

β) Screening test for the tolerance of grapevine vitroplants to *Botrytis cinerea*

The implementation of the test consisted in inoculating the leaves of plantlets, which were being cultured in vitro on micropropagation medium, by depositing 20 µl of a suspension of conidia, containing 1.10^{-4} conidia/ml (200 conidia per deposition) in malt/glucose medium on their upper surface. The plantlets, four different leaves of which had thus been inoculated, were then cultured in a climate chamber (photoperiod: 16 h day, 8 h night; temperature: 24°C; humidity: 70%). Two days after the inoculation, the leaves of row 4, i.e. the youngest leaves, were inspected (the necroses and macerations which were present were counted using a camera which was linked to a television monitor) and extracted with methanol in order to assay the resveratrol which had been synthesized in response to the *Botrytis* attack. At 5 days, the number 2 leaves were removed for observation by fluorescence microscopy. Macroscopic observation (necroses and macerations in the leaves) and counting of the leaves exhibiting fruiting bodies of the fungus (conidiophores) were also performed on the number 3 leaves. Finally, at 9 days, leaves which were interacting with the fungus, and which had been removed from three different plants, were extracted with methanol in order to assay the resveratrol.

γ) Induction of biotic stress by depositing a suspension containing *Botrytis cinerea* spores on vitroplant leaves -test of tolerance to *Botrytis*

This direct confrontation test was performed using the technique which has already been described (cf. the preceding paragraph). The same applies to the observations which were made. Four vitroplants were used for each variety or transformed clone and, in the case of each of these vitroplants, three leaves which had developed in rows 2, 3 and 4 were inoculated with the conidial suspension (200 conidia in 20 µl of malt/glucose medium). 12 inoculations were therefore performed for each variety or clone and the following analyses and observations were made:

→ Two days after inoculation:

- inspection of the leaves in row 4 for foliar symptoms (fungal maceration zone or necrotic spots, which consist of small blackish brown regions which are located around the fungal spores, or without visible symptoms) and,
- assay of resveratrol in the same leaves.

→ Five days after inoculation:

- inspection of the leaves in row 3 for foliar symptoms with counting of those leaves which carry conidiophores (fruiting bodies of the fungus) and
- fluorescence microscopic inspection of the leaves in row 2 in order to locate the regions of resveratrol synthesis.

→ Nine days after inoculation:

- assay of resveratrol in three leaves which are derived from three different plants and which are interacting with the parasite.

For each experimental series, three *Vitis vinifera* varieties having different susceptibilities to *Botrytis* attack on their leaves were studied: Folle blanche (susceptible), Pinot noir (moderately tolerant) and Ugni blanc (tolerant), together with the stock-vine 41B (tolerant) as well as four of its transformants: one having inserted supernumerary copies of the genes

encoding a stilbene synthase (13 kb insert), i.e. clone 55-3, and the three others representing transformants harbouring the PMs PR10-1 promoter-*vst1* gene construct, that is clones 145-2, 145-3 and 145-6.

b) Results obtained

The results for the foliar symptoms observed at 2 or 5 days after the inoculation are presented in Tables 7 and 8 and the results for the assays of resveratrol, related either to dry weight or to chlorophyll content, are presented in Tables 9 and 10.

Table 7: Macroscopic observations of vitroplant leaf/*Botrytis cinerea* interactions at 2 days

Variety tested	Number of No. 4 leaves observed in 4 different plants in which the following symptoms appear		
	Regions of maceration	Necrotic spots	No visible symptom
Folle blanche 280	4	1	0
Pinot noir 386	3	4	0
Ugni-blanc 479	1	2	1
41B	0	4	0
Pct 55-3	2	3	0
Pct 145-2	0	3	1
Pct 145-5	0	1	3
Pct 145-6	1	1	2

Legend to Table 7:

- Regions of maceration: broad and diffuse region in which *Botrytis* is rapidly destroying the plant cells. These regions have a light brownish beige colour.

- Necrotic spots: very small regions centred around the fungus. These spots have a blackish brown colour.

Table 8: Macroscopic observations on the vitroplant leaf/*Botrytis cinerea* interactions at 5 days

Variety tested	Number of No. 3 leaves observed in 4 different plants in which the following symptoms appear		
	Regions of maceration	Necrotic spots	No visible symptom
Folle blanche 280	4	0	4
Pinot noir 386	3	1	2.5
Ugni-blanc 479	2	1	0.5
41B	1.5	2	0.5
Pct 55-3	2	2	1
Pct 145-2	1.5	2	1
Pct 145-5	0.5	4	0
Pct 145-6	3	2	1

Legend to Table 8:

- Regions of maceration: broad and diffuse region in which *Botrytis* is rapidly destroying the plant cells. These regions have a light brownish beige colour.

- Necrotic spots: very small regions centred around the fungus. These spots have a blackish brown colour.

Table 9: Results of the HPLC assays of resveratrol which were carried out on different grapevine varieties which had been interacting with *Botrytis cinerea* for 2 days

Variety tested	Resverat. $mg.g^{-1}$ of chloroph.	Resverat. $\mu g.g^{-1}$ of dry weight
Folle B280	5.1	101
Pinot N386	4.3	102
Ugni B479	3.9	86
Stock-g41B	5.7	112
Pct 55-3	4.6	118
Pct 145-2	6.9	170
Pct 145-5	2.2	83
Pct 145-6	4.3	107

Legend to Table 9:
- Resverat.: resveratrol; chloroph.: chlorophyll
Folle B 280: Folle blanche 280; Pinot N 386: Pinot noir 386; Ugni B 479: Ugni-blanc 479; Stock-g41 B: Stock-vine 41B

Table 10: Results of HPLC assays of resveratrol which were carried out on different grapevine varieties which had been interacting with *Botrytis cinerea* for 9 days

Variety tested	Resveratrol $mg.g^{-1}$ chlorophyll	Resveratrol $\mu g.g^{-1}$ of dry weight
Folle blanche 280	4.0	38
Ugni-blanc 479	31.7	145
Stock-vine 41B	11.9	59
Pct 145-5	602.1	2558

→ Two days after inoculation:

The observation related to the youngest leaves (row 4). Regions of maceration (colonization by the fungus) were observed in almost all cases, apart from 41B and clones 145-2 and 145-5. The susceptible grapevine varieties exhibited these regions to a greater extent than did the tolerant varieties: Folle blanche and Pinot noir exhibited 4 and 3 regions, respectively, as against only 1 in the case of the Ugni blanc variety. Only the 145 transformants (PMs PR10—1 promoter-*vst1* gene) and the Ugni blanc gave leaves without visible symptoms. Necrotic regions, which are a plant defence reaction, appeared mainly in 41B and its transformants and, in the case of the grapevine varieties, in Pinot Noir and Ugni blanc.

If these observations are compared with the resveratrol assays (Table 9), it is not possible to establish any correlation since, when related to dry weight, the contents of resveratrol in these leaves are comparable with a value of approximately 100 $\mu g.g^{-1}$ of dry material. In all the vitroplants examined, the contents settle at values of between 4 and 5 $mg.g^{-1}$ of chlorophyll.

It is only the stock-vine 41B and, in particular, transformant 145-2 which have higher values. Clone 145-5 has a lower value (approximately half the size),

A comparison can also be made with the resveratrol contents which were previously obtained at 20 hours after inducing the vitroplants with UV light (Table 6). Although the period of sampling is not comparable (20 hours in the case of the abiotic stress as compared with 48 hours in the case of the biotic stress, although the time necessary for the germination of the spores has to be taken into account), lower contents of resveratrol are generally observed after a biotic stress (half or one third, in the case of the Ugni blanc, of the amount), apart from the Pinot noir, where the content is comparable, and the Folle blanche, where the content is

approximately three times greater.

In all the samples analysed, it may be emphasized that there was a marked scattering of the values obtained in the different repeat assays which were performed. This variability between different leaves of one and the same plant, which was previously observed with the samples which had been subjected to the UV light inductions, could, in the latter case too, be due to several different factors. The most plausible hypotheses which may be mentioned are:

A wide variability between vitroplants of one and the same clone in their reaction in the face of fungal attack. The variable foliar symptoms which were obtained following inoculation with the *Botrytis* spores appear to confirm this (variability in the infection, in the physiological state of each plant, etc.).

The assay, which is performed on the whole leaf, is not representative of the variations in the synthesis of resveratrol which exist in each cell which forms the leaf. Thus, it is generally acknowledged that, in hypersensitivity reactions to a parasite, it is not all the cells of the lamina which synthesize defence molecules. It is only the cells which are located close to the regions of fungal attack which are induced to synthesize phytoalexins. Therefore, the analyses which were performed in this test only represent values which correspond to a mean resveratrol level which is present in the lamina of leaves which are interacting with the parasite. A sizeable dilution effect can therefore be produced, in particular in the case of the resistant plants, between the concentrations which are present in the cells which are induced to synthesize phytoalexin and the value which is found when the whole leaf is analysed. This effect is doubtless more marked during the early stages of expression of the plant defence reactions.

→ Five days after the inoculation:

The symptom observations show (photographic plates Nos. 1 and 2) (Figures 10 and 11) and Table 8)

that regions of maceration are formed, in relatively large numbers, in all the grapevine varieties, in the stock-vine and in the transformants which were studied. These maceration regions are often associated with the presence of conidiophores (fruiting bodies of the fungus). Of the grapevine varieties and the control stock-vine 41B, the Folle blanche, which is the species which is most susceptible to *Botrytis*, shows attacks on all the leaves studied, and also conidiophores. If a hierarchy in the severity of the symptoms is established, the Pinot noir comes next, followed by the Ugni blanc and finally 41B. However, in the case of these two latter varieties, conidiophores only developed on one single half-leaf. As far as the transformant clones are concerned, three clones gave reactions which were more or less similar: 55-3, 145-2 and 145-6. Only clone 145-5 exhibited good tolerance to the parasite since a region of maceration only developed on one half-leaf and no conidiophores were visible.

On the other hand, most of the leaves of this clone reacted to the attack by forming necrotic regions. An example is also depicted in photographic plate 2 (Figure 11).

In order to verify whether these results corresponded satisfactorily to the marked differences in the induction of resveratrol synthesis, and therefore to the expression of gene(s) encoding a stilbene synthase, leaves which were inoculated with the *Botrytis* spores were inspected by fluorescence microscopy (filter unit A: excitation filter of from 340 to 380 nm; stop filter at 425 nm). Under these conditions, chlorophyll fluoresces red while resveratrol fluoresces bluish white or a darker blue depending on its concentration. Two grapevine varieties, i.e. Folle blanche (susceptible) and Pinot noir (moderately tolerant), the stock-vine 41B (tolerant) and one of its transformants, i.e. 145-5 (PMs PR10-1 promoter- *vst1* gene construct), were studied by this method. The photographs obtained from these observations are presented in photographic plate 3

(Figure 12). Striking differences can be seen. In the case of the Folle blanche, the parasite mycelium (black in the photograph) has developed and has colonized the tissues. Practically no bluish cell is visible. In the case of the Pinot noir variety, a bluish region can be observed, which region forms a barrier around and in the region of inoculation and incipient colonization by the fungus. The growth of the mycelium is retarded, if not blocked, by this barrier, even if a tissue maceration process has already been instituted. A more intense blue coloration also exists in the veins. In the region studied in the case of the control stock-vine 41B, scattered cells, which are distributed throughout the lamina, display a light blue fluorescence with an intensity which is greater in the veins. No fungal colonization process has been instituted, and scattered necrotic regions, which are limited to a few cells, can be seen. With regard to the 41B which is transformed with the PMs PR10-1 promoter-*vst1* gene construct, the results are spectacular in the case of clone 145-5. While incipient colonization of the tissues by the fungus has been instituted (black region), a cell barrier, which is synthesizing resveratrol, has formed very rapidly around this region thereby preventing it from being extended. Blue cells are still visible in the region of fungal maceration. Furthermore, many of the cells of the lamina of the leaf which are not in contact with the fungus have themselves synthesized resveratrol. The veins also display an intense blue coloration.

These observations demonstrate that a correlation therefore exists between the intensity of the foliar symptoms which developed after inoculating vitroplants with *Botrytis* spores and the content of stilbenic phytoalexin in the leaves. The most tolerant plants are those which display a resveratrol synthesis which is distributed over a large part of the lamina. This is the case with transformant 145-5, which harbours a gene encoding a stilbene synthase under the control of the PMs PR10-1 promoter, which promoter was isolated

from lucerne.

→ Nine days after inoculation:

The symptom observations demonstrated that the Folle blanche vitroplants were particularly susceptible to *Botrytis* attacks in this test as well. At nine days, they are all infested with the fungus and their chlorophyll is markedly degraded in most cases. With regard to the Ugni-blanc variety and the stock-vine 41B, it was not possible to observe any difference in expression of the foliar symptoms. Generally speaking, the symptom observation results which were obtained at 5 days are encountered once again while being a little more developed in the case of the regions of maceration. On the other hand, most of the leaves which exhibited conidiophores have necrosed.

4 varieties were analysed for their content of resveratrol: the Folle blanche (susceptible), the Ugni-blanc (tolerant), the 41B (tolerant) and 145-5 (41B transformed with the PMs PR10-1 promoter-*vst1* gene). Nine days after inoculation, 145-5 still did not exhibit any significant symptoms of *Botrytis* attack. The results of the resveratrol content analyses are presented in Table 10. When expressed in µg of resveratrol $.g^{-1}$ of dry weight or in $mg.g^{-1}$ of chlorophyll, the resveratrol concentrations can be used to rank the varieties in the following manner, proceeding from the lowest value to the highest: Folle blanche < 41 B < Ugni-blanc < the transformed 41B strain 145-5.

The concentration differences are very substantial since the transformant 145-5 has a value which is almost 43 times greater than that of the 41B control (per g of dry weight) and 50 times greater if it is expressed per g of chlorophyll. The assays therefore provide good confirmation of the fluorescence microscopy assessments that were made at five days (see photograph plate 3, Figure 12).

With regard to the Ugni-blanc and the 41B, Table 10 shows that the former has approximately 3 times more resveratrol than the latter whichever units are

selected. However, these two varieties react in an identical manner in terms of foliar symptoms. This similarity in tolerance can be explained by factors other than the synthesis of resveratrol. It must also be noted that, under the conditions of the test, the plant/*Botrytis* confrontation is particularly favourable to the latter. The environmental conditions which exist in a container in vitro ensure that, after approximately 20 days of culture, all the vitroplants, whatever their nature, are infected with the fungus.

c) Conclusions on the genetic transformation of grapevine and analysis of the efficiency of the promoters studied

It was not possible to regenerate plants containing the constructs which comprised the *vst1* gene under the control of the 35S promoter or its derivatives from the experiments on 41B (stock-vine hybrid *V. Vinifera x V. Berlandieri*) embryogenic cells which were transformed with *Agrobacterium tumefaciens* harbouring the different constructs. It was not therefore possible to obtain any strong constitutive activity over the whole of the plant.

By contrast, grapevine plants which had incorporated the PMs PR10-1-*vst1* gene and 13kb insert constructs (*vst1* and *vst2* genes under the control of their native promoters) were obtained. It was possible to compare these genetically transformed plants with the control (41B untransformed) and multiply them by micropropagation.

Few differences were observed, as compared with the untransformed controls, in the concentrations of resveratrol which were obtained, under UV light stress (biotic stress), in the different transformants which were prepared with the 13 kb insert, in which two genes, i.e. *vst1* and *vst2*, both of which are under the control of their native promoters, are present in the sequence used as insert.

By contrast the results demonstrate that the chimeric PMsPR10-1-*vst1* gene construct enables an

approximately 50-fold overexpression of the phytoalexin resveratrol (product of reaction catalysed by a stilbene synthase) to be achieved, in the presence of a biotic stress caused by *Botrytis cinerea*, at 9 days after infection. These plants then demonstrate the best tolerance when they are compared either with the control or with the transformants which were obtained with the 13 kb insert.

Thus, transformation with the construct which links the inducible lucerne promoter PMs PR10-1 to the stilbene synthase genes enables the stilbene synthase genes to be overexpressed in grapevine plants in response to a stress such as pathogen attack.

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CLAIMS

1. Nucleic acid which comprises the sequence of the promoter for a lucerne PR protein linked to at least the sequence of a gene encoding a stilbene synthase.

2. Nucleic acid according to Claim 1, characterized in that the promoter for a lucerne PR protein is a promoter which can be induced in plants, in a tissue-specific manner or not, by a biotic or abiotic stress.

3. Nucleic acid according to one of Claims 1 and 2, characterized in that the sequence of the promoter for a lucerne PR protein is selected from the group comprising:

a) the IND S1 sequence,

b) the sequence which is of a fragment of the IND S1 sequence and which has a promoter sequence effect in plants, and

c) a sequence exhibiting at least 80% homology with the IND S1 sequence.

4. Nucleic acid according to Claim 3, characterized in that the sequence of the promoter for a lucerne PR protein exhibits at least 90% homology with the IND S1 sequence.

5. Nucleic acid according to Claim 3, characterized in that the sequence of the promoter for a lucerne PR protein exhibits at least 95% homology with the IND S1 sequence.

6. Nucleic acid according to one of Claims 1 to 5, characterized in that the sequence of the gene encoding a stilbene synthase is selected from the genes isolated from groundnut, orchid, grapevine and pine genomes.

7. Nucleic acid according to Claim 6, characterized in that the sequence of the gene encoding a stilbene synthase is the sequence of a gene encoding a grapevine stilbene synthase.

8. Nucleic acid according to Claim 7, characterized in that the sequence of the gene encoding a grapevine stilbene synthase is a sequence selected from:

a) the *vst1* gene

b) the *vst2* gene.

9. System for expressing a stilbene synthase gene in plants, characterized in that it comprises at least one nucleic acid according to one of Claims 1 to 8.

10. System for expressing a stilbene synthase gene in plants according to Claim 9, characterized in that the system is a vector.

11. Expression vector according to Claim 10, characterized in that the vector is a plasmid.

12. Expression system according to one of Claims 9 to 10, characterized in that it can be transferred into *Agrobacterium* strains.

13. Expression system according to one of Claims 9 to 12, characterized in that it can be induced in plants by a biotic or abiotic stress.

14. Expression system according to Claim 13, characterized in that the biotic stress is a parasite attack.

15. Expression system according to Claim 14, characterized in that the parasite is a bacterium, a yeast, a fungus or a virus.

16. Expression system according to Claim 15, characterized in that the parasite is *Botrytis cinerea* or *Plasmopora viticola*.

17. Expression system according to Claim 13, characterized in that the abiotic stress is a mechanical wound.

18. Expression system according to Claim 17, characterized in that the mechanical wound is caused by an insect.

19. Expression system according to Claim 17, characterized in that the mechanical wound is caused by a physical phenomenon such as wind or frost.

20. Plant cell which is transformed with a system or a vector according to one of Claims 9 to 19.

21. Cell according to Claim 20, characterized in that it is a grapevine cell.

22. Process for obtaining a cell according to one of Claims 20 and 21, characterized in that a plant cell is

transformed using a microbiological method including a system or vector according to one of Claims 9 to 19.

23. Process for obtaining plants which express a stilbene synthase gene, characterized in that cells of the said plants are transformed using a system or a vector according to one of Claims 9 to 19, the cells expressing the said gene are selected and a plant is regenerated from these cells.

24. Plant comprising an expression system according to one of Claims 9 to 19.

25. Plant comprising cells according to one of Claims 20 and 21.

26. Plant which is obtained by implementing a process according to one of Claims 22 and 23.

27. Plant according to one of Claims 24 to 26, characterized in that it is a plant of agricultural interest.

28. Plant according to Claim 27, characterized in that the plant is grapevine.

FIGURE 1: Scheme for isolating the PMs PR 10-1 promoter

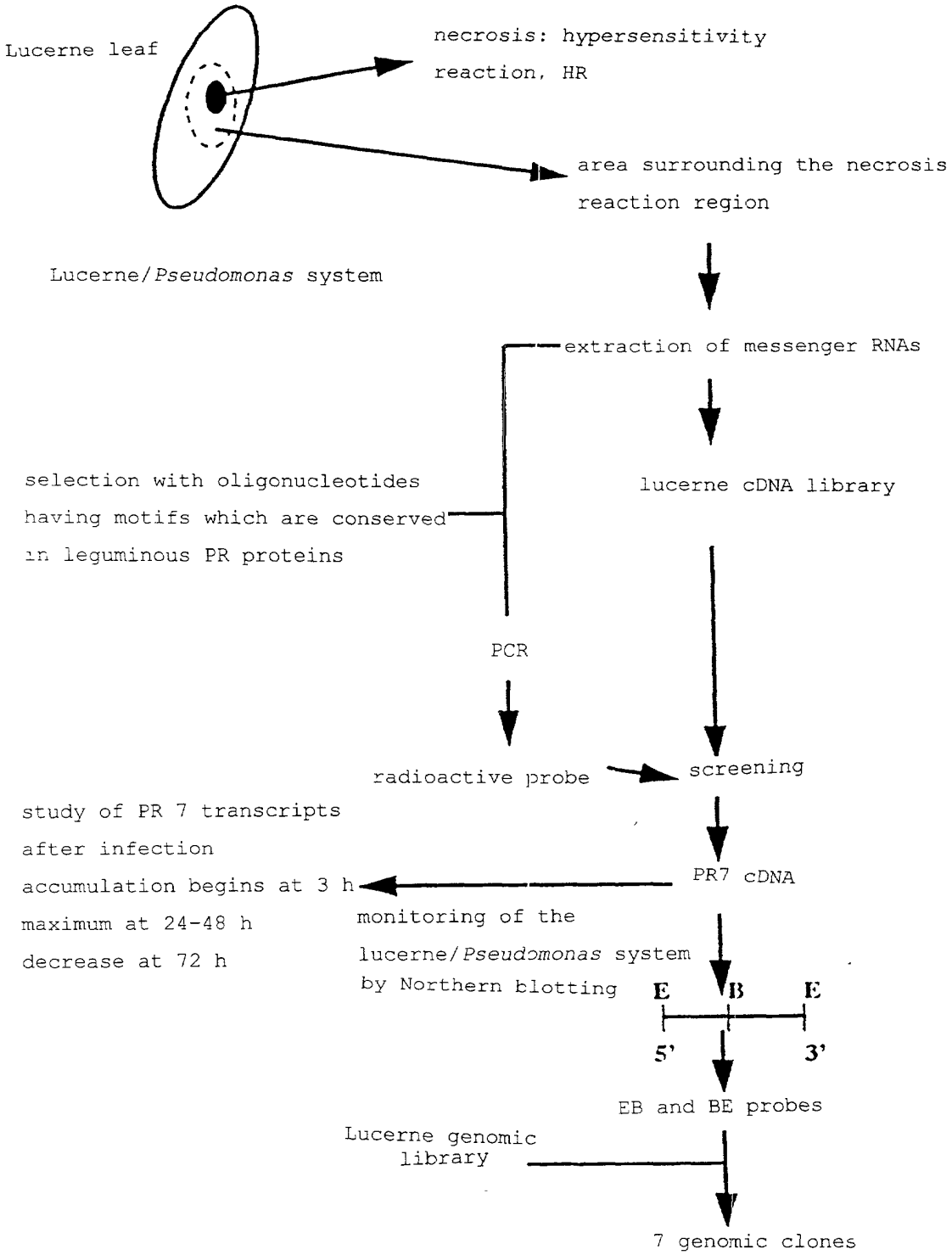

FIGURE 1a: Scheme for isolating the Ms PR10-1 promoter (continued)

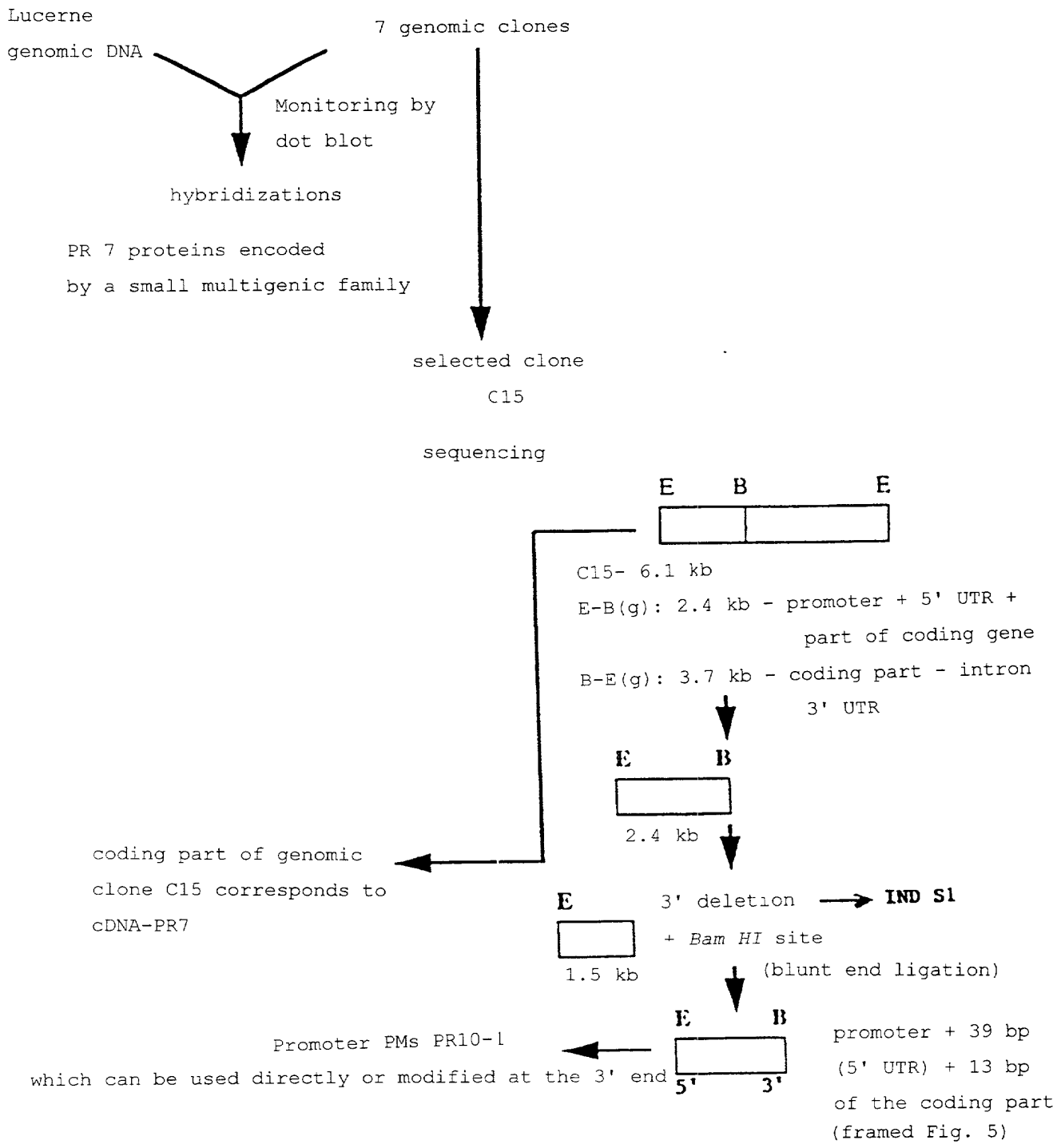

FIGURE 2

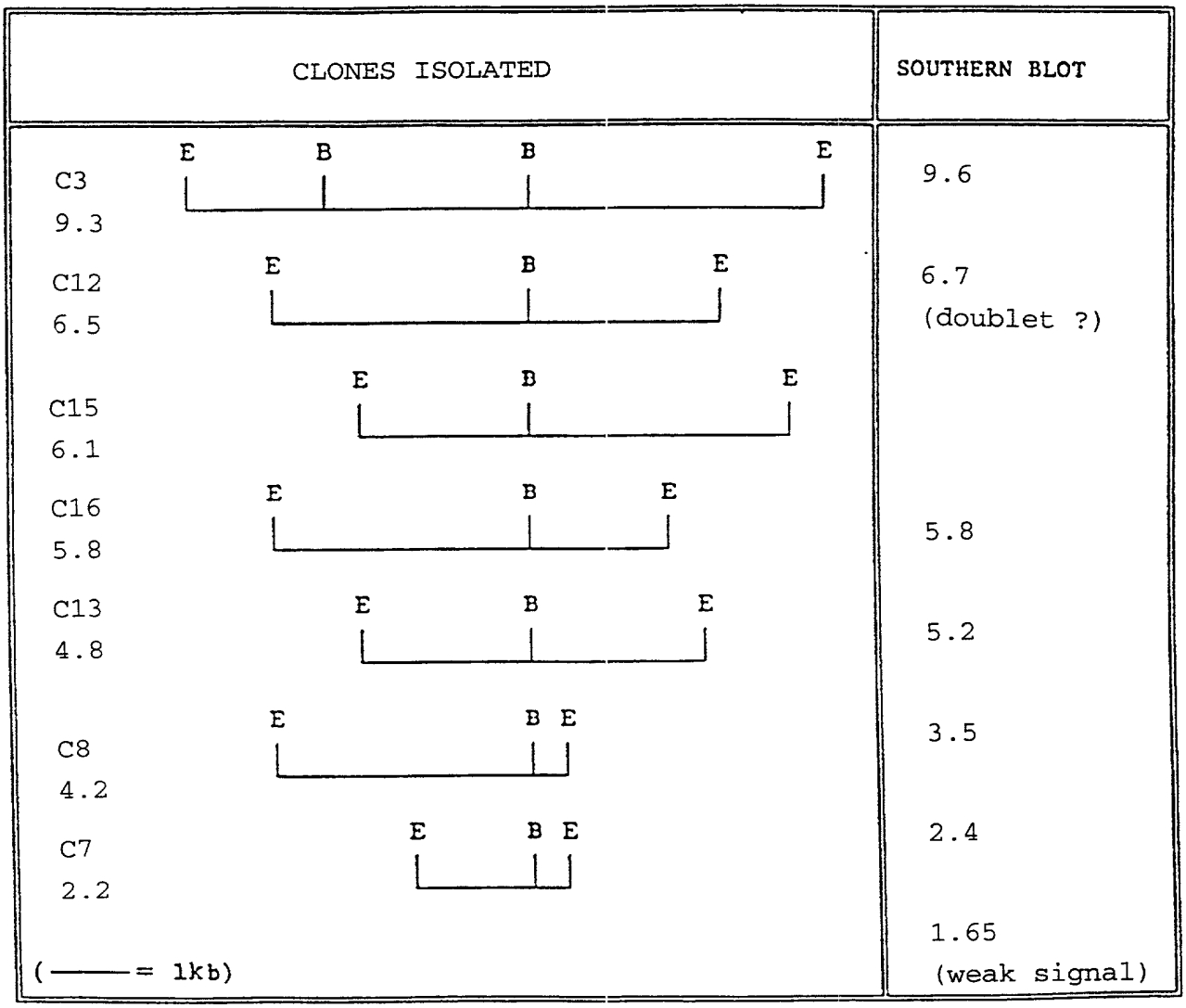
CLONES ISOLATED
SOUTHERN BLOT
E B B E
C3
9.3
9.6
E B E
C12
6.5
6.7
(doublet ?)
E B E
C15
6.1
E B E
C16
5.8
5.8
E B E
C13
4.8
5.2
E B E
C8
4.2
3.5
E B E
C7
2.2
2.4
1.65
(weak signal)
(——— = 1kb)

FIGURE 3

gaattcttcaaaaaaaaagttgcccttgagaaactaataagttaataaactaagacctctaa
aaaaaaagttaataaactaatatgaatattctctaaacaaaaaataaaactaagaagaatat
attttgcttatttaccagaaaaatactttgcttagtcaaaagaagaagaatattgtgaatta
atttgatactgatgatttttaaagctgtagatatttacgtatttagttaaaaaaatacaatt
attatatatttaattggtgtgtctattcaagtgtttaacttaagttgaggtttattcttatg
ttactaagttggagtggagaagaagactatttgcttgggaggaggaacgcccagtagaatgt
gttattatttttttatttttttgtaaggagtagagtgtgttatgttgcttgaataatttttt
ttgtaggataatgtattagacaaataaatttggaaacacgaccctgtcaaagagtacacggt
aaaggggtggtatacaaaagagtgcgtcgctctattcttcaggtcatttggtttgctacag
tttaggaaatttgggaggaaagaaataacagactgtataacgtcaaagaatgctcggttatt
caggtggtagataagattaagtttcttgcttttgcatgggtgaaggcaaagtttgcttctct
tccattcaattaccatgggtggcggcttagtccgtttaccatactggacataggctaagagt
ttttctttttctcgtttttccattacaagttctttatgtaaatactgttttgactttggtgtt
cttcccttagtacaccttgtgctaggaaggactattttgatttggtaatatatttcatttta
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aataaatgattaaattgaaaataaagataaataactaaaatgttttctataattaagttaag
agatgaaatatgtaattttcccaattatatattatgtaagtttttatttatttatatacgt
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tcttttcctataaatacaagactcaactccactcataaatcacacaggcaaacaattaactt
cttaatagtttgttatttcacacattag

FIGURE 4

*GATCCGGCTTC*AATTGAGGAAATTAGAAACGCTCAACGTGCCAAGGGTCCGGCCACCATC
CTAGCCATTGGCACAGCTACTCCCGACCACTGTGTCTACCAGTCTGATTATGCTGATTAC
TATTTCAGAGTCACTAAGAGCGAGCACATGACTGAGTTGAAGAAGAAGTTCAATCGCATA
Tgtaagtatatatattcatgcattaattcttacattcacaacatttctatacatatacga
gtgtgctattaagtgagggtcacctccaagtgaatgaatgtttcaagcttagagaatagc
ttttagctaaattactttaggaaacttgaaaatcattttacatcagtaaccgatattcct
ttcatttgattgtaagggcttgaagagctgttctttgaatcatgtagcattgctagctat
aattaagaataacctttttataatttcttcaatgttaaatgcatgttgatcatcttcaaga
atatactatatgactagtcgttggaaaactaatgtgttcatcttatttcttttacaggGT
GACAAATCAATGATCAAGAAGCGTTACATTCATTTGACCGAAGAAATGCTTGAGGAGCAC
CCAAACATTGGTGCTTATATGGCTCCATCTCTCAACATtACGCCAAGAGATTATCACTGC
TGAGGTACCTAAACTTGGTAAAGAAGCAGCATTGAAGGCTCTTAAAGAATGGGGTCAACC
AAAGTCCAAGATCACCCATtCTTGTATTTTGTACAACCTCCGGTGTAGAAATGCCCGGTG
CAGATTACAAACTCGCTAATCTCTTAGGCCTTGAAACATCGGTTAGAAGGGTGATCTTGT
ACCATCAAGGTTGCTATGCAGGTGGAACTGTCCTTCGAACTGCTAAGGATCTTGCAGAAA
ATAACGCAGGAGCACGAGTTCTTGTGGTGTGCTCTGAGATCACTGTTGTTACATTTCGTG
GGCCTTCCGAAGATGCTTTGGACTCTTTAGTTAGGTCAAGCCCTTTTTGGTGATGGGTCA
GCAGCTGTGATTGTTGGATCAGATCCAGATGTCTCCATTGAACGACCCCTCTTCCAACTT
GTTTCAGCAGCACAAACGTTTATTCCTAATTCAGCAGGTGCTATTGCGGGTAACTTACGT
GAGGTGGGACTCACCTTTCACTTGTGGCCTAATGTGCCTACTTTGATTTCCGAGAACATA
GAGAAATGCTTGAATCAGGCTTTTGACCCACTTGGTATTAGCGATTGGAACTCGTTATTT
TGGATTGCTCACCCTGGTGGCCCTGCAATTCTTGATGCAGTTGAAGCAAAACTCAATTTA
GAGAAAAAGAAACTTGAAGCAACAAGGCATGTGTTAAGTGAGTATGGTAACATGTCTAGT
GCATGTGTCTTTGTTTATTTTGGATGAGATGAGAAAGAAATCCCTAAAGGGGGAAAAAGC
TATCCACAGGTGACGGATTGGATTGGGGGTACTATTCGGTTTTGGGCCAGGCTTGACCAT
TGAGACCGTTGTGCTGCATAGCGTTCCTATGGTTACAAATTGAgtggaaaacggtaagag
aaatgatataggggacatgtcttattgtattatcagaggaggtgctacgaaagatatgta
catgtatcttcaaagttaataattagtactcctaaatcttttattcctatcctaacattg
agggattgtaatttagtgattgttggagggtgcagtcacgtcaggcaagtggatgaaact
gcaagtgcttgtcattctgttatcgggggatcatccatcacactggcggccgctcgagca
tgcat

FIGURE 5

gaattcttcaaaaaaaaagttgcccttgagaaactaataagttaataaactaagacctct
aaaaaaaaagttaataaactaatatgaatattctctaaacaaaaaataaaactaagaaga
atatattttgcttatttaccagaaaaatactttgcttagtcaaaagaagaagaatattgt
gaattaatttgatactgatgatttttaaagctgtagatatttacgtatttagttaaaaaa
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tattcttatgttactaagttggagtggagaagaagactatttgcttgggaggaggaacgc
ccagtagaatgtgttattatttttttatttttttgtaaggagtagagtgtgttatgttgct
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tcaaagaatgctcggttattcaggtggtagataagattaagtttcttgcttttgcatggg
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gtaaatactgttttgactttggtgttcttcccttagtacaccttgtgctaggaaggacta
ttttgatttggtaatatatttcattttaacctcttaaaaaaaaatcaggaaaagaaaaag
ataaaggtcggaagtgttacctgattataaaataaatgattaaattgaaaataaagataa
ataactaaaatgttttctataattaagttaagagatgaaatatgtaattttcccaattat
atattatgtaagttttttatttatttatatacgttgttttgctttgaaatttgagtggtc
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agtccaaataataatatagttttctttaaaaataatatcatccaaactcatacattaaaa
atattattcaaatttatgtcacgtcacaatgagaaaaaatggcccaacgaccttgtatta
cacatcatcgtcatcatcatctaaagtctaaacaatacatcttcttttcctataaataca
agactcaactccactcataaatcacacaggcaaacaattaacttcttaatagtttgttat
ttcacacattaggggccagATGGAGGATCCGGCTTCAATTGAGGAAATTAGAAACGCTCA
ACGTGCCAAGGGTCCGGCCACCATCCTAGCCATTGGCACAGCTACTCCCGACCACTGTGT
CTACCAGTCTGATTATGCTGATTACTATTTCAGAGTCACTAAGAGCGAGCACATGACTGA
GTTGAAGAAGAAGTTCAATCGCATATgtaagtatatatattcatgcattaattcttacat
tcacaacatttctatacatatacgagtgtgctattaagtgagggtcacctccaagtgaat
gaatgtttcaagcttagagaatagcttttagctaaattactttaggaaacttgaaaatca
ttttacatcagtaaccgatattcctttcatttgattgtaagggcttgaagagctgttctt
tgaatcatgtagcattgctagctataattaagaataacctttttataatttcttcaatgtt
aaatgcatgttgatcatcttcaagaatatactatatgactagtcgttggaaaactaatgt
gttcatcttatttcttttacaggGTGACAAATCAATGATCAAGAAGCGTTACATTCATTT
GACCGAAGAAATGCTTGAGGAGCACCCAAACATTGGTGCTTATATGGCTCCATCTCTCAA
CATtACGCCAAGAGATTATCACTGCTGAGGTACCTAAACTTGGTAAAGAAGCAGCATTGA
AGGCTCTTAAAGAATGGGGTCAACCAAAGTCCAAGATCACCCATtCTTGTATTTTGTACA
ACCTCCGGTGTAGAAATGCCCGGTGCAGATTACAAACTCGCTAATCTCTTAGGCCTTGAA
ACATCGGTTAGAAGGGTGATCTTGTACCATCAAGGTTGCTATGCAGGTGGAACTGTCCTT
CGAACTGCTAAGGATCTTGCAGAAAATAACGCAGGAGCACGAGTTCTTGTGGTGTGCTCT
GAGATCACTGTTGTTACATTTCGTGGGCCTTCCGAAGATGCTTTGGACTCTTTAGTTAGG
TCAAGCCCTTTTTGGTGATGGGTCAGCAGCTGTGATTGTTGGATCAGATCCAGATGTCTC
CATTGAACGACCCCTCTTCCAACTTGTTTCAGCAGCACAAACGTTTATTCCTAATTCAGC
AGGTGCTATTGCGGGTAACTTACGTGAGGTGGGACTCACCTTTCACTTGTGGCCTAATGT
GCCTACTTTGATTTCCGAGAACATAGAGAAATGCTTGAATCAGGCTTTTGACCCACTTGG
TATTAGCGATTGGAACTCGTTATTTTGGATTGCTCACCCTGGTGGCCCTGCAATTCTTGA
TGCAGTTGAAGCAAAACTCAATTTAGAGAAAAAGAAACTTGAAGCAACAAGGCATGTGTT
AAGTGAGTATGGTAACATGTCTAGTGCATGTGTCTTTGTTTATTTTGGATGAGATGAGAA
AGAAATCCCTAAAGGGGGAAAAAGCTAtCCACAGGTGACGGATTGGATTGGGGGTACTAT
TCGGTTTTGGGCCAGGCTTGACCATTGAGACCGTTGTGCTGCATAGCGTTCCTATGGTTA

FIGURE 5 (continuation 1)

CAAATTGAgtggaaaacggtaagagaaatgatataggggacatgtcttattgtattatca
gaggaggtgctacgaaagatatgtacatgtatcttcaaagttaataattagtactcctaa
atcttttattcctatcctaacattgagggattgtaatttagtgattgttggagggtgcag
tcacgtcaggcaagtggatgaaactgcaagtgcttgtcattctgttatcgggggatcatc
catcacactggcggccgctcgagcatgcat

FIGURE 6

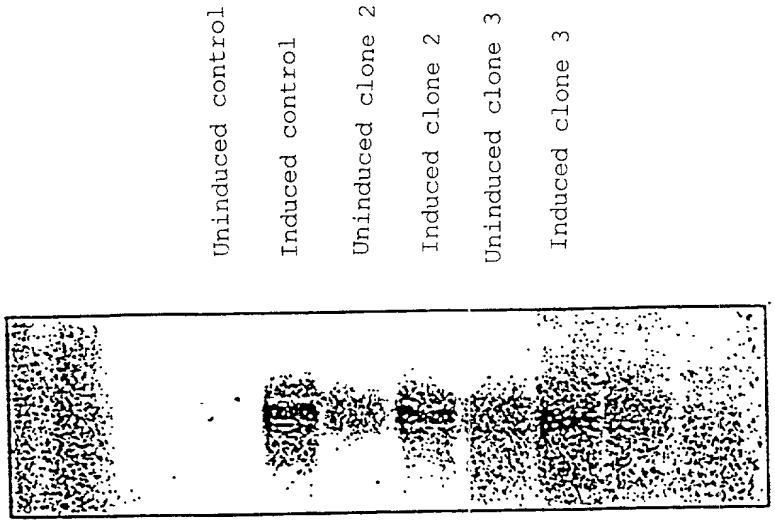

Uninduced control
Induced control
Uninduced clone 2
Induced clone 2
Uninduced clone 3
Induced clone 3

FIGURE 7

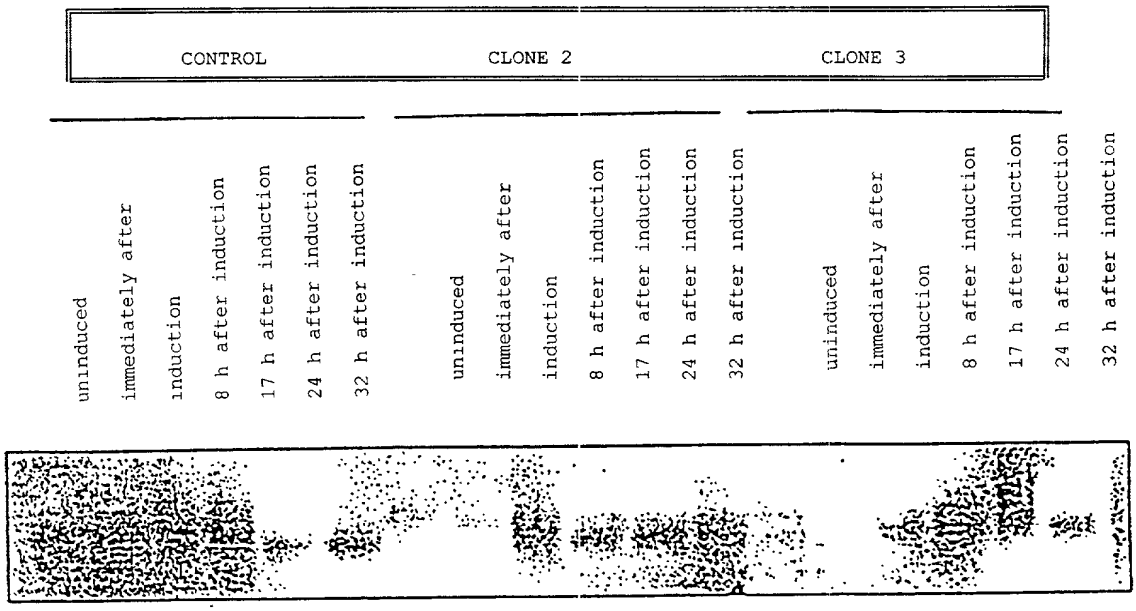

CONTROL
CLONE 2
CLONE 3
uninduced
immediately after induction
8 h after induction
17 h after induction
24 h after induction
32 h after induction
uninduced
immediately after induction
8 h after induction
17 h after induction
24 h after induction
32 h after induction
uninduced
immediately after induction
8 h after induction
17 h after induction
24 h after induction
32 h after induction

FIGURE 8

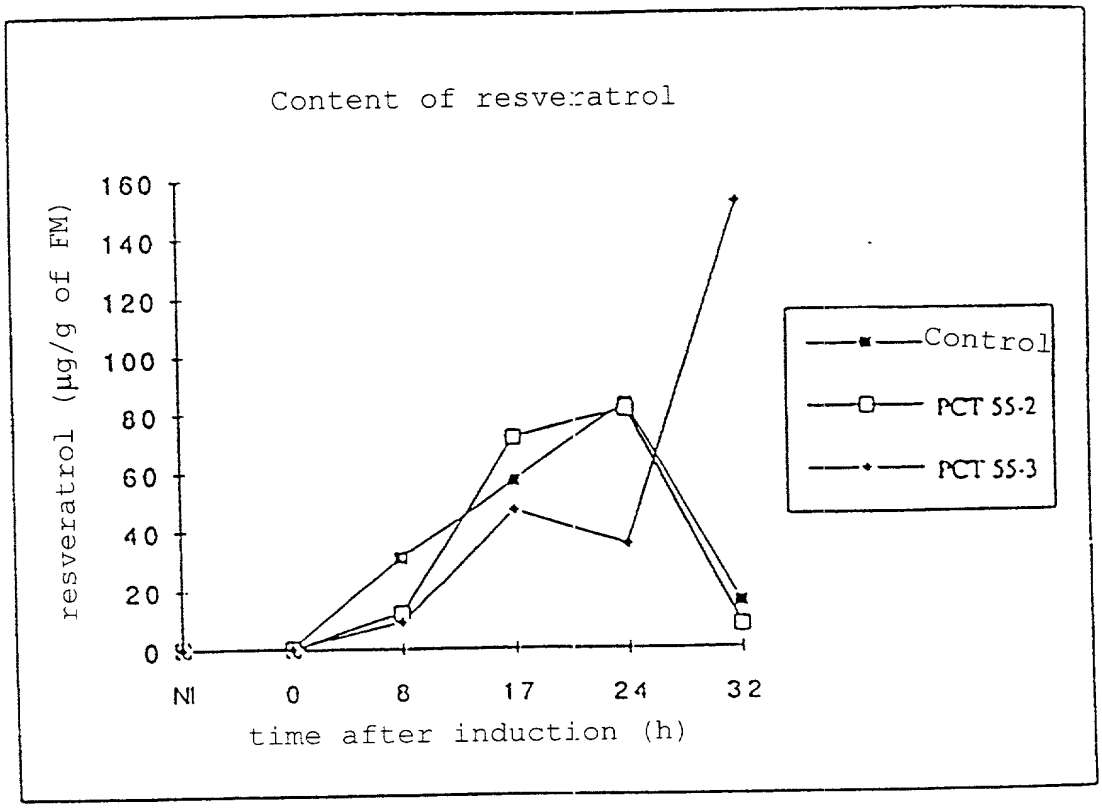

Content of resveratrol
resveratrol (µg/g of FM)
160
140
120
100
80
60
40
20
0
NI
0
8
17
24
32
time after induction (h)
Control
PCT 55-2
PCT 55-3

FIGURE 9

Inhibition of the growth of the mycelium of *Botrytis cinerea* 916 t after 7 days at 20°C

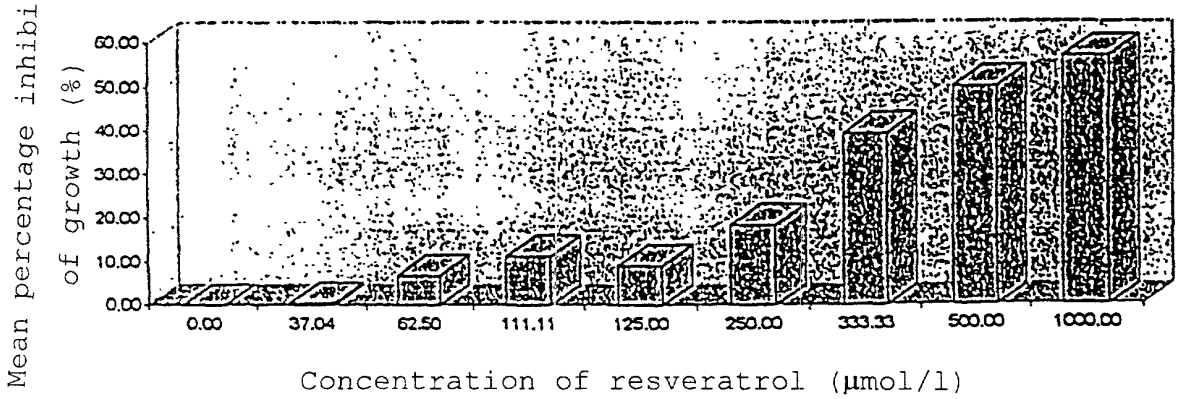


FIGURE 10: Photographic plate 1

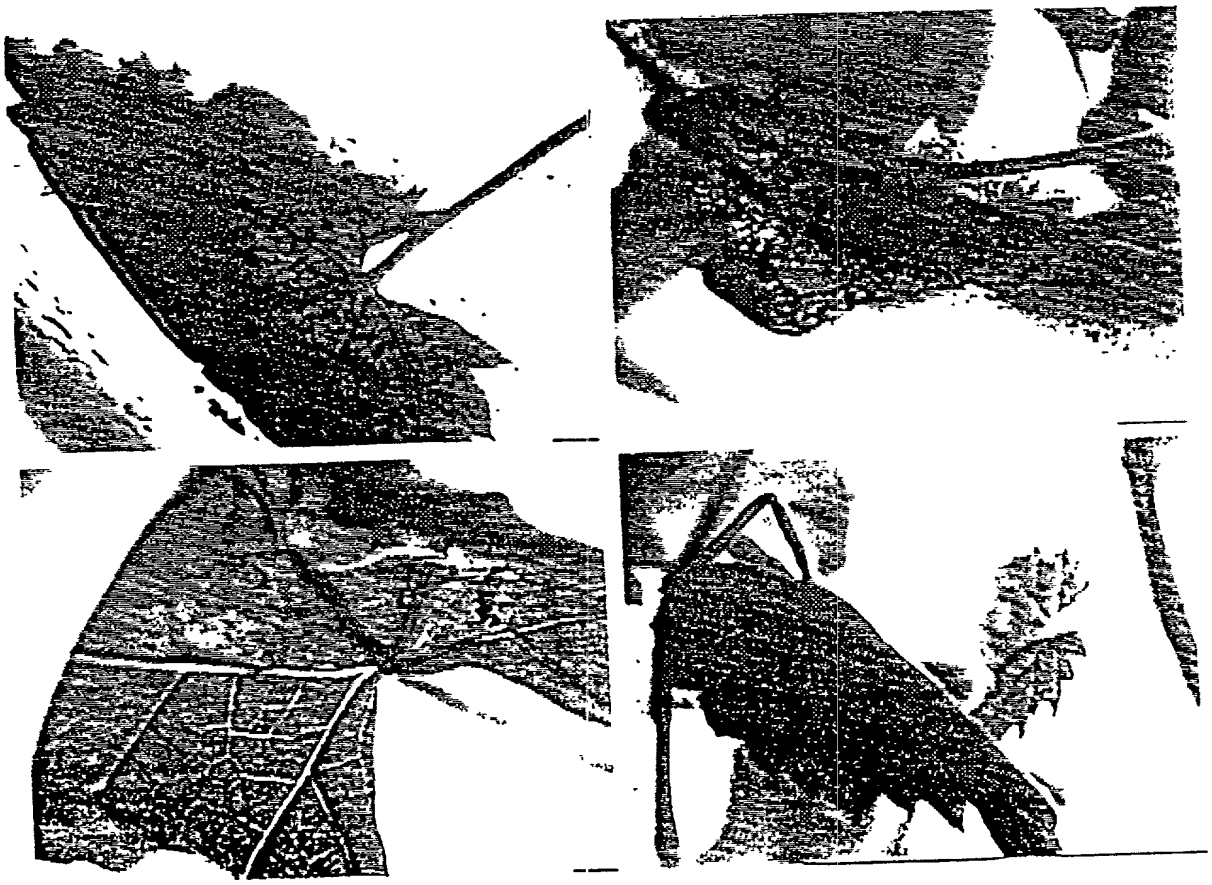


FIGURE 11: Photographic plate 2

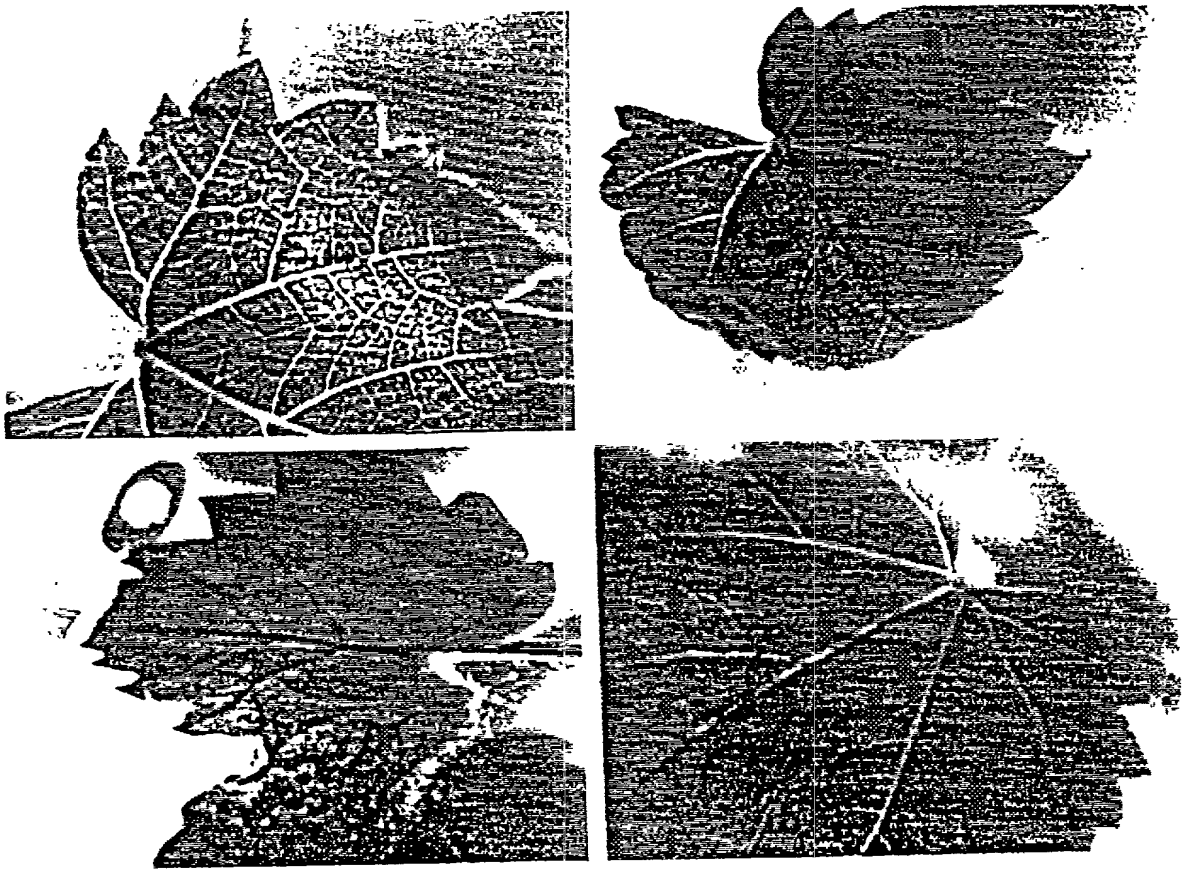
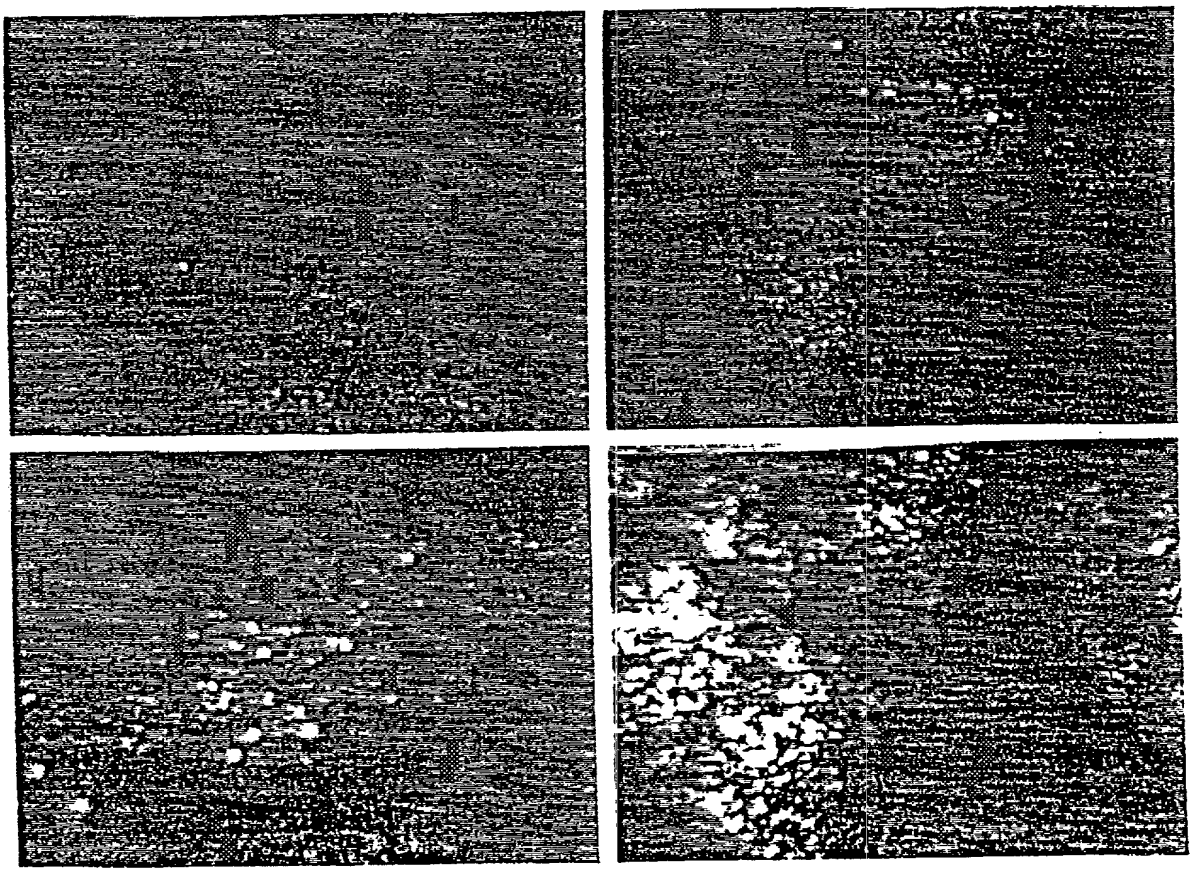


FIGURE 12: Photographic plate 3



DECLARATION FOR PATENT APPLICATION

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **NUCLEIC ACID COMPRISING THE SEQUENCE OF A STRESS-INDUCIBLE PROMOTER AND A SEQUENCE OF A GENE ENCODING A STILBENE SYNTHASE**

the specification of which (check one)

[ ] is attached hereto. [X] was filed on Feb.12 1999, as ~~United States Patent Application Serial No. or~~ PCT International Application Number PCT/FR99/00316, and was amended on _____ 19__ (if applicable)

X was filed on August 14, 2000 as U.S. Serial No. 09/622,257

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with 37 CFR § 1 56(a)

Prior Foreign Application(s) I hereby claim foreign priority benefits under 35 U S C § 119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate listed below, or § 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed

(Application No)	(Country)	(Day/Month/Year Filed)	Priority Claimed [X] Yes [ ] No
98/01742	FRANCE	13/February/1998	

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below:

Application No	Filing Date

I hereby claim the benefit under 35 U S C § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by 35 U.S.C. § 112, first paragraph, I acknowledge the duty to disclose material information as defined in 37 CFR § 1 56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

(U S Application Serial No)	(U S Filing Date)	(Status--patented, pending, abandoned)

I hereby appoint George Vande Sande, Registration No. 17,276, Burton A. Amernick, Registration No 24,852, Richard Wiener, Registration No. 18,741, Townsend M Belser, Jr , Registration No 22,956, Morris Liss, Registration No 24,510; Martin Abramson, Registration No 25,787; George R Pettit, Registration No. 27,369, Elzbieta Chlopecka, Registration No 32,767, Eric J. Franklin, Registration No. 37,134; Jeffri A Kaminski, Registration Number 42,709, and William E. Curry, Registration No 43,572, my attorneys with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon

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~~[ ] See next page for additional inventors~~

DECLARATION FOR PATENT APPLICATION

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Full name of sixth joint inventor (if any) _____

Inventor's Signature _____ Date _____

Residence Address _____

Citizenship _____

Post Office Address _____

Full name of seventh joint inventor (if any) _____

Inventor's Signature _____ Date _____

Residence Address _____

Citizenship _____

Post Office Address _____

Full name of eighth joint inventor (if any) _____

Inventor's Signature _____ Date _____

Residence Address _____

Citizenship _____

Post Office Address _____